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An overview of liquid chromatography-mass spectrometry

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AN OVERVIEW OF LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

By

George Guan

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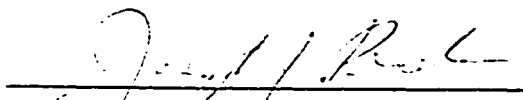
APPROVED FOR THE DEPARTMENT OF CHEMISTRY



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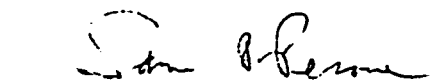

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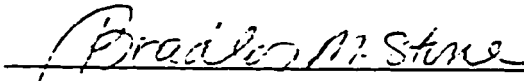

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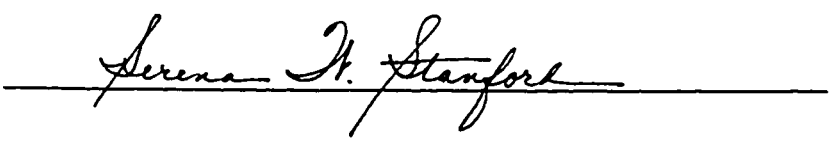

Abstract

An Overview of Liquid Chromatography-Mass Spectrometry

by George Guan

This thesis reviews the LC-MS techniques with particular attention to their applications in the analysis of amino acids and peptides. Although the development of the on-line LC-MS was inspired by the success of GC-MS, particularly in the field of biological sciences due to the wider applicability of LC, LC and MS are basically incompatible. In order to overcome the incompatibility in the conjunction of LC with MS, many successful interfaces have been designed including MBI, DLI, CF-FAB, TSP and ESP, especially CF-FAB, TSP and ESP which introduce soft ionization and therefore, LC-MS techniques have become accessible to large biomolecues. The application of LC-MS has been successful for the determination of molecular weight and sequence of peptides.

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I am very grateful to my advisor, Dr. Joseph Pesek, for his guidance, support and encouragement he has given to me over the past years. Dr. Pesek has been always ready to help me in difficult situations. His patient and friendly help made it possible for me to finish my work.

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1. Basic principles of liquid chromatography

1.1. General aspects

Chromatography is a physical method used to separate components that are selectively distributed between two immiscible phases: a mobile phase and a stationary phase. The technique is named based on the mobile phase: gas chromatography (GC) and liquid chromatography (LC).

The chromatographic separation process occurs as a result of repeated different distributions of the individual analytes in the sample while the mobile phase is flowing through the stationary phase bed. When the substance transferred from the mobile to the stationary phase is selectively adsorbed at the surface of the stationary phase, it is referred to as adsorption chromatography. On the other hand, if the component is selectively retained by dissolving in the stationary liquid phase, it is partition chromatography. In both cases the retained components are subsequently selectively removed (eluted) by passing the same or a different mobile phase over the stationary phase. In actual practice, it is difficult to obtain a separation which is exclusively due to either one or the other. There is almost always some adsorption present in even the best partition chromatography. The reverse is also true.

The conventional open column liquid chromatography is inefficient and extremely slow. While gas chromatography is much more rapid and efficient, its direct applications are limited to those samples which have a low vapor pressure and are heated without decomposition. This requirement has excluded gas chromatography from many determinations of important organic and biochemical compounds. High performance (pressure) liquid chromatography has overcome all these disadvantages. LC in this paper is the abbreviation of High Performance Liquid Chromatography.

In liquid chromatography analytes are separated based on their different distribution between a liquid mobile phase and a stationary phase. While the basic principles remained essentially the same, high-performance liquid chromatography uses small particles of uniform size and shape with a narrow pore size distribution, together

with chemical modification of the surface functionality. Unlike traditional liquid chromatography using gravity flow or low-pressure solvent-delivery systems, the use of high-efficiency packing materials of small diameter requires high-pressure solvent pumping systems that can deliver the mobile phase at uniform and reproducible flow-rates.

A basic configuration of an LC instrument is given in Figure 1.1.1. The appropriate solvents (mobile liquid phase) from the reservoirs are allowed to enter the mixing gradient chamber where a homogeneous mixture is obtained. A pump capable of maintaining high pressures draws the solvent from the mixing chamber and pushes it though the column. The sample is injected through a port into the high pressure liquid carrier stream between the pump and the column. The separation takes place on the column. The separation is monitored with a flow-through detector. [1]

1.2. The columns

1.2.1. Column materials and configuration

The heart part of the chromatographic system is the column. In general, an LC column is a cylindrical tube made of stainless-steel to withstand high pressures and the chemical action of the mobile phase. Where the inertness of the stainless steel is insufficient, tantalum or titanium may be used. Glass-lined metal columns combine the advantages of the inertness of glass and the mechanical strength of the metal, but they have not found much favor. Fused-silica columns find use in capillary or open tubular LC. Polyethylene columns are not suitable for the internally pressurized systems but find use in the radially compressed systems (Radially compression system is not discussed in this paper.).

Straight columns are preferred and are operated in the vertical position. The dimensions of the columns are generally determined by the instrument configuration, the convenience of packing and the size of packing particles. The column is packed with porous solid supports, which can either act as a solid phase or as a base on which the

stationary phase is built. The columns are usually packed by means of a slurry packing technique, using specialized equipment. Most column lengths range from 10 to 30 cm; short, fast columns are 3 to 8 cm long. For exclusion chromatography, columns are 50 to 100 cm long [2].

1.2.2. Column maintenance

The column needs some maintenance routinely. The most harmful factors for the column life are particulate matter and strongly retained impurities coming from either the sample or the mobile phase. Cleaning the column and filling it with a mobile phase is generally advisable. However, when the performance of a column has fallen below acceptable limits, the stationary phase can often be cleaned by pumping a series of solvents with a variety of porarites. It must also be mentioned that most buffers are insufficiently soluble in pure organic solvents; therefore, washing a buffer-filled reverse phase column with only pure methanol might result in clogging. For a normal-phase column the following solvent sequence may be used: heptane, chloroform, ethyl acetate (acetone), ethanol (methanol), water, methanol, chloroform, heptane. For a reversed-phase column the sequence may be: water, methanol, dichloromethane, methanol, water, 0.1M sulphuric acid, water. Stationary phase cleaning will prolong the lifetime of the column and prevent clogging.

To prolong the life of analytical columns, guard columns are often inserted ahead of the analytical column where they act as both physical and chemical filters. Guard columns are relatively short (usually 5 cm) and contain a stationary phase similar to that in the analytical column. They protect the analytical column from particulate contamination that arises from a poorly filtered mobile phase or from degrading sample-injection valves. A guard column extends the lifetimes of the expensive separation column by capturing the strongly retained sample components and preventing them from gradually contaminating the upper layers of the analytical column. Guard columns are by design expendable and are periodically repacked, replaced, or reconditioned. Guard

columns inevitably will result in a loss of efficiency. If the loss in resolution due to the extra-column effect cannot be tolerated, an in-line filter designed to remove particulates should be placed in front of the column. [3]

1.2.3. Standard Column

Many LC separations are done on columns with an internal diameter of 4 to 5 mm. Such columns provide a good compromise between efficiency, sample capacity, and the amount of packing and solvent required. Column packings consist of particles that are uniformly sized and mechanically stable. Particle diameters lie in the range 3-5 μm, occasionally up to 10 μm or higher for preparative chromatography. For preparative separations larger-bore columns are more useful.

It is common knowledge that the efficiency of the packed column in chromatography depends on the diameter of the particles used for the packing. However the high pressure drop across the columns increases rapidly with the column length and inversely with the particle diameter. In view of the very large back pressure it is concluded that the smaller packing particle diameters are, the shorter the columns have to be. Thus, the optimum length of columns in HPLC has generally been proportional to the particle diameter, e.g. the 10 μm particles are used to pack 10-25 cm columns, while the column length for 5 μm and 3 μm particles is generally 5-10 cm and 3-5 cm, respectively. For a conventional standard column, the injection volume is about 5-20 μl, and the flow-rate is 500-2000 μl/min.

1.2.4. Microcolumns

The column diameter has very little influence on the efficiency of a column. Beyond about 4 mm i.d., there is or no effect of increasing column diameter. This is probably the reason for the 4 mm i.d. columns becoming nearly universal among the commercially available pre-packed columns. However, the column diameter has an influence on the signal of a sample. Decreasing the internal diameter of the column by a

factor of two increases the signal of a sample component by a factor of four, the square of the change in diameter, which makes the narrow-bore columns ideally suited for trace analysis, particularly in biological samples where the availability of the sample may be limited.

The main advantages of the narrow-bore column LC system appears to be the considerable reduction in the solvent consumption and the consequent saving of the high-purity solvents and expenditure in laboratories where large numbers of analyses are performed. Another advantage in small-bore columns is their suitability for more detection modes, such as direct liquid introduction interfacing to mass spectrometer detection.

Miniaturization can refer to the decrease in column length, in particle diameter or in column internal diameter; however, the discussion in this paper is restricted to miniaturization in column internal diameter. Micro-LC and microcolumn is used throughout this paper to refer to LC systems or columns with reduced internal diameter. Four types of microcolumns have been used in applications, i.e., open-tubular columns, drawn packed capillaries, packed microcapillaries, and microbore columns. [4,5]

In the open-tubular LC columns, the mobile phase flows through an open tube (fused silica, 5-25 μm ID, and 1-20m in length), and the open tube wall is where the stationary phase is deposited. The application of homogeneous stationary phases is used in most cases. The production of homogeneous stationary phase films in narrowbore tubes is still a problem. The injection volume for an open tubular column is often less than 1 nl, and the flow-rate is less than 0.1 μl/min.

Drawn packed capillaries are produced by drawing out a glass capillary packed with LC packing material (5-10 μm particles) to a column with an internal diameter of 50-100 μm ID and a length of 2-20 m.

The microcapillary packed columns are 0.2-0.5 mm ID, and 0.1-0.5m in length of fused-silica tubes packed in a conventional way with an LC packing material. These

microcapillary columns are packed very efficiently so that plate numbers in excess of 10^5 are often achievable. The injection volume is about 0.1 μl and the flow rate is 0.5-2 μl/min.

Microbore columns are similar to conventional standard columns except miniaturization in both particle diameter and column internal diameter. The internal diameter of a microbore column is about 0.5-2mm and 0.1-1 m long. The injection volume of a microbore column system is about 0.5-2 μl. A flow rate of 1-50 μl/min is normally used in the microbore column system.

1.3. Separation modes

One of the major advantages of LC over other separation techniques is to be found in the several different mechanisms by which the chromatographic separation may be achieved. These mechanisms or modes of operation make it possible to achieve separations by liquid chromatography for diverse samples. The conventional classification of separation modes in LC is that of normal-phase chromatography, reversed-phase chromatography, ion-exchange chromatography, and size-exclusion chromatography. Although the modes of LC will be discussed individually in the following sections, in reality most LC techniques are the result of mixed mechanisms.

1.3.1. Normal-phase chromatography (NP)

Normal-phase systems are characterized by use of a polar stationary phase and a nonpolar mobile phase and are used for the analysis of relatively polar compounds. Dry silica is highly polar and will strongly adsorb even traces of polar impurities such as water from mobile-phase solvents and when the polarity of the analytes increases, they show a tendency be retained on the column. Therefore, polar compounds give tailing and asymmetric peaks. The introduction of new, bonded-phase silicas has greatly improved the peak appearance of polar compounds. Today, The use of bare silica as a stationary phase has significantly declined since the introduction of the bonded phases.

Among many stationary phases such as alumina and chemically bonded stationary phases, silicagel still is the adsorbent used in most applications. The functional characteristic of silicagel is the silanol (-Si-OH) groups at the surface. The different electrostatic forces between the the dipole of the silanol (-Si-OH) groups at the surface of silicagel and the dipoles in the different analyte molecules result different retention time for analyte molecules. The retention time of a component from a NP-LC not only depends on the typical stationary phase but also is influenced by the composition of the mobile phase. Solvents play an important role in the mass distribution ratio, and therefore it has strong influence on the retention of a typical analyte on the silicagel surface. Solvents can be rated from weak to strong according to their polarity. For example, pentane and hexane are weak solvent, chloroform and dichloromethane are moderate solvent, but propanol and methanol are considered as strong solvent. In general, the retention of an analyte in a NP-LC can be altered by changing the composition of the solvent system.

The most significant drawback of bare silicagel is that it is highly polar and will strongly adsorb even traces of water in the eluent. The retention of an analyte will be affected when water is strongly adsorbed on the silicagel. Therefore, in order to obtain a reproducible result, having control of the water content on the silicagel surface becomes extremely important. One way to achieve the control of water content on the silicagel surface is to condition the stationary phase [10,11].

1.3.2. Reversed-phase chromatography (RP)

Reversed-phase LC is used for the analysis of non-polar analytes. Its stationary phase has the reversed property compared to the normal-phase LC. Reversed-phase systems consists of chemically modified silicagel or other non-polar packing materials such as styrene-divinylbenzene copolymers used as stationary phase and aqueous-organic solvent mixtures used as the polar mobile phase.

In reversed-phase LC, the silicagel surface is modified by reacting it with an organochloro- or organoalkoxysilanes, e.g.:

$$\equiv \text{Si-OH} + \text{Cl-Si}(\text{-CH}_3)_2\text{-R} \longrightarrow \equiv \text{Si-O-Si}(\text{-CH}_3)_2\text{-R} + \text{HCl}$$

In the pH range 2.5-8 the siloxane bond is stable in organic and aqueous solvents. In the bonded group, another important part is that the R-groups can be varied, which gives specific applications for reversed-phase. Some of the R-groups that can be bonded by the above mentioned pathway are octyl (C_8), octadecyl (C_{18}), phenyl, n-propylamine, alkyldiol, alkyl-N+$(CH_3)_3$, and phenylsulfonate ($\text{-}C_6H_4\text{-}SO_3^-$). The bonded-phase materials are used in a variety of LC applications, and the R-groups with octyl (C_8) and octadecyl (C_{18}) are the most important ones. The bonded-phase materials offer many advantages such as the relative stability, the short equilibration time and the applicability with gradient elution. However, the phase bonding reactions cann't modify all silanol groups at the surface of the silicagel and the reproduction of the reaction is also uncontrollable, which is the major drawbacks of the bonded-phase materials. Since there are still about 40% of Si-OH groups not be modified on the silicagel surface, the bonded-phase materials may only be applicable within certain pH range, otherwise they may give specific adsorption with basic analytes. It has been reported that the non-polar packing material, styrene-divinylbenzene copolymers can be used as a stationary phase over a wider pH-range.

Since the contact between the analyte and the bonded-phase material is weak nonspecific Van der Waals interactions, in RP-LC the solubility of the analyte in the solvent becomes more important than in adsorption chromatography. In general, the solvent consists of water or aqueous buffers and an organic modifier (methanol, acetonitrile, etc.). When the polarity of the analytes increases, their retention decreases. The retention of non-ionic analytes can be adjusted by changing the percentage and type of the organic modifier. Considerable attention is given to automated optimization of RP-LC separations. When the components in a mixture span a relatively wide polarity

range, the separation can be achieved in a shorter time and with better efficiency by the application of a gradient elution program, where the modifier content of the mobile phase is continuously increased with analysis time.

The retention of ionogenic analytes is dependent on the pH of the mobile phase, and in a certain pH range, the ionogenic analytes in their ionic form show little retention to the stationary phase. The buffer is therefore frequently used to suppress the protolysis of the ionogenic analyte. One example is the use of phosphate buffers that are extensively applied due to their wide pH range and good buffer capacity.

When the analytes contain certain functional groups that may undergo protolysis the pH of the mobile phase plays a important role. However, in the analysis of acidic or basic compounds adjustment of the pH is not always successful. One applicable method is to add an organic lipophilic ionic compound as a counter-ion which will form ion-pairs with ionic analytes. The ion-pairs are well retained on the reversed-phase material, e.g.:

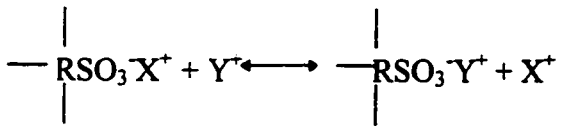
$$R_4N^+ + A^- \longrightarrow [R_4N^+\ A^-]$$

Quaternary ammonium compounds and sulphonic acids with a long alkyl chain are widely used counter-ions The possible mechanism is that part of the ion-pairing agent is adsorbed at the surface of the reversed-phase material and actually modifies the surface which then acts as an ion-exchanger. Since the interactions are a weak reactions, column conditioning is obligatory. [10]

1.3.3. Ion-exchange chromatography (IEC)

Ion-exchange chromatography is used in the analysis of ionized or ionizable compounds. It is very useful in the separation of biomolecules such as amino acids, nucleic acids, and proteins by taking advantage of electrostatic (charge-charge) interactions. The ion exchange phase consists of a polymer or silica support backbone to which the ion exchange functional groups are attached. These bound functional groups consist of an ion and a counterion, the latter being exchangeable with an ion of the same sign in the solute. Ion exchange media used to exchange cations bear negatively charged

groups and are most often sulfonate based. Ion exchange media used to exchange anions bear positively charged groups, and usually consist of quaternary ammonium groups. The mechanism of ion-exchange chromatography is based on the following equilibrium (for a cation exchanger):



where X^+ is the stationary phase counterion and Y^+ is analyte cation. The difference in the type of counterion, the ionic strength, pH, and temperature will significantly change the retention time of the analyte molecules. In addition, complexing agents such as citrate buffers or EDTA (ethylene diamine tetraacetic acid) are sometimes added to the mobile phase to influence the separation [12,13].

1.3.4. Size-exclusion chromatography (SEC)

Size exclusion or gel permeation chromatography (SEC or GPC) is different in mechanism from most other types of chromatographic methods that we have considered, since it is essentially noninteractive. Separation occurs on the basis of size of the solute molecules, and is not due to intermolecular forces or interactions. The column consists of a stationary phase with pore structure of controllable size. Smaller molecules, which can enter the pores, will be retarded strongly while larger molecules which cannot enter the pores will elute more quickly. Molecules of intermediate size will, of course, experience moderate retention. The method is applied for the separation of bio-macromolecules in the molecular-weight range of 10^3 - 10^7 and for the characterization of the molecular weight distribution of the various oligomers. Solvent selection is relatively simple due to the noninteractive nature of the separation mechanism. A single solvent is required to dissolve the sample and does not participate in partition. The method is fast, and high efficiency separations are possible between narrow molecular weight ranges. The

technique is also applied as a physical method to determine the molecular weight distribution of polymeric materials [14].

1.4. LC detectors

Various LC detector are available at the present time. The most popular detectors used in HPLC are ultraviolet-visible photometers or spectrophotometers. These devices measure the degree to which a sample solution will absorb ultraviolet or visible light. Chromophoric groups on molecules (such as carbonyl, multiple carbon-carbon bonds, aryl groups) will absorb energy in the uv region. The absorption results in the excitation of the electronic states, and corresponds to a jump in electronic energy level. Chemical systems consisting of long, conjugated multiple bonds tend to absorb radiation in the visible region by a similar mechanism as the uv-active functionalities. Ultraviolet detectors vary in sophistication from a simple photometer to a modified diode-array spectro-photometer.

The sensitivity of the ultraviolet detector is in large measure a function of the absorptivity of the sample. A sample that strongly absorbs radiation will have a lower minimum detectable concentration. Typically, one can detect uv-active compounds down to the 10^{-10} g/ml level. The detector is relatively insensitive to temperature and flowrate variations, and is linear over four or five decades. Since it depends upon the measurement of a solute property rather than a bulk property, it is ideal for gradient elution methods. It is not affected by a changing mobile phase composition unless, of course, the mobile phase contains a chromophore. Chromophoric mobile phases are generally not usable with the uv detector. The major disadvantage of this detector is its selective response only to compounds which contain chromophoric groups.

Refractive index detectors are the second most widely used detectors in HPLC. They are universal in application since they respond to the bulk property of refractive index of the eluting stream. The response is provided by the change in total solution refractive index caused by the separated solute in the carrier stream. The refractive index

detector is much less sensitive than the uv detector although it has a minimum detectable concentration about 10^{-7} g/ml. The ultimate sensitivity of the device is dependent on the magnitude of the refractive index difference between the mobile phase and solute component.

The refractive index detector is very sensitive to changes in operating temperature and must usually be thermostatted to provide optimal performance. It is also sensitive to changes in mobile phase flowrate since flow changes will disrupt the temperature control. It is not practical to use this detector with gradient elution methods, since the mobile phase refractive index is continually changing.

The fluorimetric detector is a specific and highly sensitive device applicable to fluorescent solutes, or to solutes which can be rendered fluorescent by chemical derivatization. Fluorescence is based on the emission of photons by electronically excited molecules. It is a process that follows the absorption of a photon. The fluorescence detector detects the intensity of emitted radiation in fluorescence. The limits of detection from the fluorescence detector are lower than for absorption. The detection limits can be achieved as low as 10^{-11} g/ml. The major drawback of the fluorescence detector is the limited number of compounds that fluoresce. However, the number of fluorescing species can be enlarged by preliminary treatment of samples with reagents that form fluorescent derivatives. Fortunately, many substances or their fluorescent derivatives of biological interest can be sensitively detected.

Electrochemical detectors can provide sensitivities higher than those obtained using uv absorption and fluorescence. The detection limits can be achieved as low as 10^{-12} g/ml in general. Electrochemical detectors are based on the measurement of electrical current resulting from the oxidative or reductive conversion of an analyte at an electrode surface. Electrochemical detectors are specific and concentration sensitive detectors. They are useful for solutes which are easily oxidized, in high conductivity mobile phases such as buffer solutions.

There are other detectors available, such as infrared absorption, conductivity, radioactivity and NMR. A new, universal and promising detector, which has been becoming a popular detector for LC, is the mass spectrometry [3,15,16].

2. Introduction to mass spectrometry

2.1. General aspects

Mass spectrometry is one of the most important physical methods in analytical chemistry today. A particular advantage of mass spectrometry, compared with other molecular spectroscopies, is its high sensitivity. The high sensitivity can be traced to the electron multiplier detector used in mass spectrometers. Moreover, it gives rich information on an analyte such as the molecular weight and the structure of the analyte. A MS is the instrument used to produce ions that are sorted in the mass analyzer according to their mass-to-charge (m/z) ratio and detected. The mass spectrum is a graph of ion abundance vs. mass-to-charge ratio. Extreme selectivity can be obtained by selectively monitoring on the most significant ions, which are important for quantitative trace analysis. Mass spectrometry provides one of the few methods that is entirely suitable for the identification or quantitative measurement of trace amounts of chemicals. The useful application of mass spectrometry has been considerably extended by coupling with chromatographic techniques, initially with gas chromatography (GC/MS) and more recently with liquid chromatography (LC/MS).

The mass spectrometer nowadays is a highly sophisticated and computerized instrument. The processes that occur in a mass spectrometer are: 1) sample introduction, 2) ionization, 3) mass analysis, 4) ion detection and 5) data handling. In some applications automatic operation is possible. An important practical aspect of mass spectrometry is that the processes of ion formation, analysis, and detection conventionally take place in vacuum. The basic components of a mass spectrometer are shown in Figure 2.1.1.

A large number of different instrument configurations can be used to perform these functions. In mass spectrometry sample introduction systems are to permit introduction of a representative sample into the ion source with minimal loss of vacuum. Most modern mass spectrometers are equipped with four types of inlets to accommodate various kinds of samples; these include direct insertion probe, reservoir inlet, gas

chromatograph, and liquid chromatograph (in this review, the interest is limited to the liquid chromatograph as an inlet). The ionization of the analytes can be performed in a number of ways such as electron impact, chemical ionization, fast atom bombardment, thermospray, and atmospheric pressure ionization. After the production of the ions, they are analyzed according to their mass-to-charge ratio in a mass analyzer. The common mass analyzers are the quadrupole mass filter and double focusing magnetic sector. The detection of ions after the mass analysis is mostly performed by means of an electron multiplier. Efficient means to collect and handle the enormous amounts of data that are generated in the operation of a mass spectrometer are of utmost importance. Highly advanced computer programs are currently available for use in handling, interpretation and reporting the data. Considerable improvements have been made in this field in the last few years as a result of the growth in computer science and technology [17,18].

2.2. Ionization methods

There are several ionization methods. Electron impact was the first ionization method to be used routinely and is still the most widely employed method in mass spectrometry overall. It is suitable for a large number of synthetic and naturally occurring compounds but is limited by the need for sample vaporization prior to ionization. In addition, electron impact ionization does not always give molecular weight information, but this problem may be over come by the use of chemical ionization.

The introduction of fast atom bombardment (FAB) as an ionization method removed the need for sample volatilization and marked the first effective entry of mass spectrometry into the field of biopolymer analysis. LC/MS has received a boost from the introduction of methods where ionization actually takes place from solution. In particular, electrospray and ionspray techniques have extended the molecular weight limits of mass spectrometry much farther than ever before.

2.2.1. Ion-source

An ion source has the dual function of producing ions from the sample and accelerating them into the mass analyzer. In all source designs there must be an ion withdrawal and focusing system. Ions are usually removed electrostatically from the chamber and accelerated toward the mass analyzer.

The control of partial pressures in an ion source is critical to establish probabilities of molecular collisions and thus also ion lifetimes. To ensure high probabilities of ionization, the pressure in an electron-impact ion source is held at about $1x10^{-3}$ Pa (10^{-4} - 10^{-5} torr). Somewhat higher pressures will be needed in chemical ionization.

Between the ion source and detector pressures are maintained in the range of 10^{-4} - 10^{-5} Pa (10^{-6} - 10^{-7} torr) to keep the collision of analyte ions with residual gas or other analyte ions minimal. In fact, pressures below 10^{-5} Pa in intermediate modules are also unattractive as they result in too few ions of some species reaching the detector [19].

2.2.2. Electron impact ionization

Figure 2.2.1 shows an electron-impact ion source system. The electron impact ion source is a small chamber where an electron beam originating from a heated filament has been accelerated through a potential of about 70 volts into the source. Gas molecules entering the ion source interact with the electron beam. Some of these gas molecules lose an electron to form a positively charged ion whose mass corresponds to the mass of the original neutral molecule. This is the molecular ion. Many molecular ions have sufficient excess energy to decompose further to form fragment ions. Those positive ions (molecular ions and fragment ions) are drawn out to the analyzer by a small electrostatic field between the large repeller plate (charged positive) behind them and the first accelerating slit of the draw-out lens (charged negative) ahead of them. Thus, the molecular ion gives an immediate measurement of the molecular weight of the sample. The mass and abundance values of the fragment ions may be used to characterize

structural information of the neutral molecule and become characteristic properties of the neutral molecule. Together, the molecular and fragment ions constitute the mass spectrum of the sample. A mass spectrum of an compound may be used as a fingerprint which can then be compared with standard mass spectra (Figure 2.2.2).

Since a high-vacuum ion source (typically 10^{-3} Pa) is required in EI ionization process, collisions between ions and background are avoided in this way. As a result, EI mass spectra are highly reproducible and computer spectral libraries are available for data searching and identification of unknown compounds. A significant disadvantage of EI is the prerequisite of sample volatilization prior to the ionization, which is unacceptable for analysis of those nonvolatile and thermally labile compounds. Furthermore, in the EI process, the energy of bombarding electrons is generally much greater than that of the bonds which hold the molecule together, which causes extensive fragmentation obscuring the molecular ion. In addition, in EI ionization negative ions, that are produced from other electron capture processes, often have low abundance. Therefore, negative ion EI is not applicable [20,21].

2.2.3. Chemical ionization

Chemical ionization methods are probably the second most common procedure for producing ions for mass spectrometry. In this technique the sample is diluted with a large excess (perhaps 10^4:1) of a "reagent" gas before being subjected to the electron beam. Because of the relative quantities, electrons are much more likely to collide with molecules of the reagent than of the analyte, so the primary ions formed are charged molecules or fragments of the reagent gas. Since in most cases the CI ionization is based on a chemical reaction and the reaction is caused by frequent intermolecular and ion-molecule collisions, chemical ionization is generally performed in relatively high pressure ion sources, with pressures between 1 Pa and atmospheric pressure (10^5 Pa). CI ionization is usually a two-parts process. In the first part the reagent gas ions are produced by a similar fashion of an EI ionization. Then a series of ion molecule reactions occur during

the second part of the CI ionization, which will result the mass spectrum. One of the most common reagents is ammonia. In the CI ionization with ammonia, ammonia reacts with high-energy electrons to give NH^{+4}. NH^{+4} then transfer a proton to an analyte molecule M to form MH^+.

$$NH_4^+ + M \longrightarrow NH_3 + MH^+$$

The product, MH^+, is seen to be larger than its parent (M) by one proton (H^+), and is often represented by the symbol $(M + H)^+$, sometimes also designated as "QM^+", for quasi-molecular ion. Some proton affinities (PA) are given in Table 2.2.1. According to the proton affinities, the above reaction will take place only if the analyte molecule M has stronger affinity than that of the reagent gas. For example, pyridine will accept one proton from NH_4^+ and yield the protonated pyridine, but NH_4^+ will not transfer any proton to water.

Besides proton transfer in the chemical ionization, many other reaction modes between the reagent ions and sample molecules are involved in the CI ionization. Four typical reaction modes are:

charge exchange:	$M + X^{+\cdot} \longrightarrow M^{+\cdot} + X$
electrophilic addition:	$M + X^+ \longrightarrow MX^+$
anion abstraction	$AB + X^+ \longrightarrow B^+ + AX$

In CI ionization reagent ions often participate in different reactions. For example, NH_4^+ can transfer a proton to an analyte molecule to produce MH^+ or form an adduct with the analyte molecule to give MNH_4^+.

In CI ionization both the sample and the reagent gas are introduced into the ion source via inlets that are tightly sealed to maintain the pressure within the ion source. Unlike electron-impact ionization, chemical ionization can be made to produce useful ion currents of positive as well as negative ions representative of different samples.

Another often used reagent is methane. A two-parts process occurs. In the first part the reagent gas can undergo secondary ion-molecule interactions in addition to its primary electron impact reaction as shown below:

1. Primary electron impact reaction. In this step the reagent gas, CH_4 is ionized by an electron-impact ionization in the source:

$$CH_4 + e^- = CH_4^+ + 2e^- (CH_3^+, CH_2^+, ...)$$

2. Secondary ion-molecule reactions. Primary reagent ionization is followed by secondary ion-molecule reactions in which the primary ion reacts with additional reagent gas molecules to produce a stabilized reagent ion plasma:

$$CH_4^+ + CH_4 = CH_5^+ + CH_3$$

$$CH_3^+ + CH_4 = C_2H_5^+ + H_2$$

The second part of the chemical ionization process occurs when a reagent ion (CH_5^+ or $C_2H_5^+$) encounters a sample molecule (MH). The reagent ion and sample molecule react via any of several modes:

1. Proton transfer reaction: $CH_5^+ + MH = CH_4 + MH_2^+$
2. Charge exchange (charge transfer): $CH_4^+ + MH = CH_4 + MH^+$
3. Electrophilic addition: $CH_5^+ + MH = (CH_5 + MH)^+$
4. Anion abstraction (hydride abstraction): $CH_3^+ + MH = CH_4 + M^+$

The resulting mass spectrum, of course, includes peaks for the reagent (e.g., CH_4^+, CH_5^+, $C_2H_5^+$, etc.), as well as fragments formed from the analyte. Other commonly used reagent gases include iso-butane, helium, and argon. The reagent ions, being smaller than the analyte, cause little spectral interference, and the major advantage of chemical ionization is the simplicity of the spectrum.

Many compounds fail to give a molecular ion under electron-impact ionization, but chemical ionization is fundamentally a low-energy process which results less molecular fragmentation and intense quasi-molecular ions indicating the molecular weight of the sample. Examination of the chemical ionization spectrum illustrates both

the advantages and the disadvantages of chemical ionization. The quasi-molecular ion is formed with little energy under chemical-ionization conditions and is very stable so that molecular weight information is obtained much more readily than from the electron impact spectrum. Unfortunately, this same stability minimizes structurally informative fragmentation so that chemical ionization is often complementary to electron impact ionization as a tool in structural analysis.

The ion source for chemical ionization is similar to that for simple electron bombardment. The demands on the pumping system, however, are more severe, usually requiring separate pumps for the ionization region and the analyzer since it is necessary to maintain the reagent pressure at a level that is considerably higher than can be tolerated in the mass analyzer [20,21,22,23].

2.2.4. Fast atom bombardment ionization

Both electron impact and chemical ionization require the sample to be in the vapor phase. With many compounds, especially those of limited volatility, this requirement for vaporization prior to ionization may lead to thermal degradation so that a useful spectrum cannot be obtained. The need of less volatile molecules to be separated by liquid chromatography rather than by gas chromatography has led to the development of new methods of ionization that do not require sample volatilization.

The earliest of these methods employed energetic particle bombardment as a mean for both volatilization and of ionization. Among those methods, the best known and most widely used one is fast atom bombardment (FAB) [24]. Figure 2.2.2 shows a typical FAB source. In this technique, a beam of fast moving neutral argon or xenon atoms (argon is often used, but the more expensive xenon is more effective due to its greater mass which results in higher momentum.) directly strike a thin sample film deposited in a clean metal probe tip. The sample is usually dispersed in a few drops of glycerol, diethanolamine and other rather polar, low-volatility solvents with low vapor pressure

which easily dissolve organic compounds yet do not evaporate in a vacuum. Since a monolayer of material is completely sputtered in a matter of seconds, it is essential that the sample surface be continuously renewed. The bombarding argon beam not only ionizes the solvent (for example, glycerol (G)), but also produces intense thermal effects whose energy is dissipated through the outer layers of the sample which causes molecules and ions to detach from these surface layers and form a dense gas just above the sample surface. Subsequently, glycerol ions react with the surrounding glycerol molecules to produce $(G_n + H)^+$ as reactant ions analogous to the chemical ionization method discussed in an earlier section. The sample undergoes proton transfer, electrophilic addition, or charge transfer to produce pseudomolecular ions such $(M + H)^+$, $(M - H)^-$, and $(M + G + H)^+$ within the plasma. These ions are then extracted by a slit lens system designed to collect ions. Depending on the voltages employed on those slit lens, positive or negative ions may be extracted into the mass analyzer just as in chemical ionization. More recently, the use of neutral beams has been replaced by the use of a beam of more energetic ions such as Cs^+.

The major advantage of this ionization technique is that the sample is not volatilized in any inlet system prior to ionization. Instead, the sample is deposited (in solution) on the tip of a metal probe which is inserted, via a vacuum lock, into the ion source for analysis. In fast atom bombardment, the sample is usually dispersed in a few drops of glycerol or similar low-volatility liquid. This has the advantage that as molecules are removed from the surface, they will be replaced by diffusion from within the body of the glycerol; thus the sample can be examined continuously over a longer period of time.

The introduction of fast atom bombardment has achieved the extension of mass spectrometry to the analysis of a wide range of thermolabile and ionic materials as well as biopolymers such as peptides, oligosaccharides, and oligonucleotides. Fast atom bombardment is a relatively mild ionization process so that fragment ions are generally of low abundance [25,26,27]. Figure 2.2.3 shows a mass spectrum of dipalmitoyl

phosphatidyl choline obtained using a fast atom bombardment source. G_nH^+ ions are matrix oligomers.

2.2.5. Thermospray ionization

More recently major advances have been made in the ionization processes in which sample ions are produced directly from solution. Obviously, these processes are important in the effective coupling of liquid chromatography with mass spectrometry. All of these methods-- thermospray, electrospray, and ion spray -- are based on the dispersion of sample solution as a mist of small charged droplets. The difference among those methods lies in the various ways in which the charged droplets are produced.

In the thermospray (TS) ionization process (Figure 2.2.4), a volatile electrolyte, usually ammonium acetate, is added to an aqueous solution of the sample. The sample solution with ammonium acetate flows through a narrow heated capillary. The capillary walls are hot enough to vaporize a substantial portion of the liquid flow so that the remainder of the sample (solution) is sprayed from the capillary exit into the ion source. Although the solution is overall electrically neutral, statistical fluctuations ensure that each tiny droplet bears a slight excess positive or negative charge from the added ammonium acetate. As the the droplets decrease in size due to evaporation, the surface charge density increases until the droplets become unstable and explode to form a number of smaller droplets. Finally, the field due to the excess charge is large enough to cause the desorption of ionized sample molecules from the smaller droplets.

The mixture of solvent vapor, sample ions, and ions from the added electrolyte inside droplets in the ion source bears a resemblance to the plasma in a conventional chemical-ionization source. As a consequence, the spectra obtained in thermospray ionization have a number of similarities to chemical ionization spectra. In addition, solvent molecules may react with sample ions to form addition complexes. In the absence of electron collisions thermospray ionization is another relatively mild ionization process, so that in many cases only ions indicative of the molecular weight are seen and

structurally informative fragment ions are absent [29,30,31,32].

2.2.6 Electrospray and ion-spray ionization

In the electrospray ionization process[33,34,35,36], a flow of sample solution is pumped through a narrow-bore hypodermic needle held at a potential of a few kilovolts relative to an opposing metal plate (Figure 2.2.5). Charging of the liquid occurs and it emerges from the needle as a mist of very fine, charged droplets. Unlike thermospray, this spraying process takes place in the atmosphere and the whole process can be referred to as an example of atmospheric pressure ionization

As in thermospray, these charged droplets then decrease in size due to both evaporation and explosions until they are small enough for ionized sample molecules to desorb from the droplet surface. This part of the process also occurs at atmospheric pressure, so that sample ions have already been desorbed by the time the spray is sampled through a small orifice into the vacuum system of the mass spectrometer for analysis.

Electrospray (ES) ionization is a very mild process and, unlike thermospray, there is virtually no thermal input in the ionization process. As a result, fragmentation is completely absent and only molecular weight information is available from the spectrum. Despite the lack of fragment-ion information, electrospray ionization is an exciting new technique, especially for biochemists, since it now offers by far the most accurate method for molecular weight determination of proteins and other biopolymers.

Another interesting feature of electrospray ionization spectra is that the peptide molecular ions recorded are multiply charged, and also cover a range of charge states. High molecular-weight peptides can be protonated in solution on all amino groups in the molecule, i.e., the amino acids containing amino side chains, e.g., lysine, arginine and histidine and on the amino-terminus of the peptide. Since the ions are analyzed according to their mass-to-charge ratio rather than their mass, a twenty-times protonated peptide ion of a peptide with a molecular weight of 20,000 will show a peak at an m/z-value of 1000, thereby reducing the mass range required from any analyzer. In this way, high molecular-

weight peptides can be analyzed using a quadrupole instrument with a limited mass range. This is an especially convenient feature in electrospray, since this method of ionization is able to generate ions from considerably more massive molecules than any of the other techniques discussed so far.

In the ion-spray technique (Figure 2.2.5), pneumatic nebulization of gas from an annular sheath that surrounds the spraying needle is used to input extra energy to the process of droplet formation [33]. The main advantage of the ISP interface over the ESP interface is the higher liquid flow-rates that can be accommodated by ISP. Flow-rates as high as 200 μl/min have been reported. The higher flow-rates are possible as a result of the combined action of electrospray and pneumatic nebulization.

2.3. Mass analyzers

The function of the mass analyzer is to separate the ions according to their mass-to-charge ratio. Four different types of mass analyzers have been introduced in mass analysis [37,38,39]. Both sector and quadrupole mass analyzers are currently used in LC-MS interfacing. In the quadrupole analyzer, a quadrupole field is formed by four electrically conducting, parallel rods. Opposite pairs of electrodes are electrically connected. One diagonally opposite pair of rods is held at a DC component $+U_{dc}$ volts and the other pair at $-U_{dc}$ volts. An RF oscillator supplies a signal to the first pair of rods that is $+V \cos wt$ and an RF signal retarded by 180^{o} ($-V \cos wt$) to the second pair. The region between the four rods appears as oscillating hyperbolic potentials. With a correct choice of voltages, only ions of a given mass-to-charge value traverse the analyzer to the detector while ions having other mass-to-charge values collide with the rods and are lost. By scanning the DC and RF voltages and keeping their ratio constant, ions with different mass-to-charge ratios will pass successively through the analyzer. In this way the whole mass range may be scanned and a complete mass spectrum recorded.

The quadrupole is easy in use. The electric voltages are easily varied under computer control. The instrument typically is used at unit mass resolution. The quadrupole mass filter is the most widely applied mass analyzer in LC-MS.

In the magnetic analyzer, accelerated ions are constrained to follow circular paths by the magnetic field. For any one magnetic field strength, only ions with a given mass-to-charge ratio will follow a path with the correct radius to arrive at the detector. Other ions will be deflected either too much or too little. Thus, by scanning the magnetic field, a complete mass spectrum may be recorded just as with a quadrupole analyzer.

In a single-focusing magnetic sector instrument there is a lack of uniformity of ion energies, since the ions leaving the ionization chamber do not all have precisely the same velocity. The resulting spread in ionic energies of ions with same mass produces a spread in their radii of curvature in the magnetic field. The result is peak broadening and low to moderate resolution. In the presence of an electric field, only those ions that have the correct velocity pass through the slits at the end of the electrostatic sector. The ions with same mass all travel at the same velocity in the magnetic field region, so the resolution improves. The double-focusing magnetic sector instrument is also much more suitable than the quadrupole analyzer for the analysis of ions with higher mass-to-charge ratio. Detection of ions is accomplished, in both magnetic sector and quadrupole instruments, by an electron multiplier placed at the end of the analyzer. The output from the electron multiplier is then directed toward some kind of recording device, usually a data system [40].

2.4. Tandem Mass Spectrometry

An important technique in mass spectrometry that is proving to be increasingly useful in LC/MS is tandem mass spectrometry or MS/MS [41,42]. Most MS/MS instruments consist of two mass analyzers arranged in tandem but separated by a collision cell. In an MS/MS instrument, sample ions of a specified mass can be selected by the first analyzer and then introduced into the collision cell, where they collide with

neutral gas molecules. In particular, in a triple quadrupole instrument, the first analyzer is a conventional quadrupole analyzer set to transmit ions of the required mass and the collision cell is a second quadrupole analyzer that contains a collision gas held at a suitable pressure, but that is operated in what is called the RF-only mode. The use of a collision cell means that fragmentation is induced deliberately, and in a specific region of the instrument. Scanning the second mass analyzer, which follows the collision cell, will then record all the fragment ions formed from the precursor ion selected by the first analyzer. In the case of the triple quadrupole instrument, this second analyzer is a further conventional quadrupole analyzer.

MS/MS instrumentation has found increasing use in LC/MS in conjunction with soft ionization techniques such as fast atom bombardment, thermospray, electrospray, and ion spray. For example, a conventional spectrum recorded by any of these techniques will quite often show only a quasi-molecular ion indicative of molecular weight such as the ion at m/z 365. If, however, this same ion is selected by the first analyzer of the MS/MS instrument and introduced into the collision cell, it can be caused to fragment. The fragment ions produced that are structurally meaningful can then be recorded by scanning the second analyzer.

Another application of MS/MS techniques is to remove background ions, which may originate from the solvent in LC/MS, or ions due to unresolved components in the sample. If, for example, the first analyzer is set to transmit the quasi-molecular ion of interest as before, then none of the background ions or ions due to unresolved components will reach the collision cell. Because of this, the collision-induced spectrum contains only information that relates to the component of interest while other interfering ions have been eliminated. A number of other valuable experiments may be carried out with MS/MS instrumentation. For example, a characteristic fragmentation may be monitored to detect a particular compound or members of a particular class of compounds. This technique is sometimes called selected decomposition monitoring.

Other types of mass analyzers may be used in tandem to give alternative forms of MS/MS instrumentation. In particular, a collision cell may be interposed between a double-focusing magnetic sector analyzer and a quadrupole analyzer to give what is known as a hybrid instrument. Alternatively, the collision cell may be interposed between two double-focusing magnetic sector analyzers to give a four-sector instrument. A four-sector instrument is particularly appropriate when collision-induced dissociation experiments are to be carried out on ions with a mass-to-charge ratio much in excess of 1000.

3. General view of LC-MS

3.1. Advantages of LC-MS

3.1.1. Universal detection

There are many different detectors used for LC today, but the mass spectrometer is considered as the best universal detector. Most organic compounds as well as thousands of biomolecules with molecular weight up to 100,000 Da and more can be well detected by the mass spectrometer. There are two working modes which can be chosen in the operation of a mass spectrometer. In the scanning mode, a mass spectrometer produces complex mass spectra. The detailed nature of the spectrum for a molecule gives a thorough identification for a compound. The identification can be achieved by either comparison with reference spectra published or interpretation according to established rules. In contrast with the scanning mode, selected ion-monitoring mode (SIM) is used only to monitor certain chosen ions, which increases the sensitivity of the detection. The SIM mode is often used for quantitative analysis of known compounds[43,44].

3.1.2. Quantitative measurements and qualitative identification

Since the scanning mode generates full spectra which produces the highest information content, it provides not only the possibility to identify an unknown compound but also the ability to discriminate any interference. Therefore, when a mass spectrometer in the scanning operation is used as an LC detector, it allows the operator to identify the elute with a very high degree of confidence. During the selected ion-monitoring, the mass spectrometer only monitors a selected number of ions, so that the information content for a compound is reduced. However, if those selected ions have exclusive and significant characteristics for a compound, SIM mode still presents the impressive ability to discriminate against interference. One good example is that when an isotope-incorporated internal standard which has chemical similarity to an analyte is added into the analyte, the isotope-incorporated internal standard can be distinguished due to its different molecular weight and fragment ions. Moreover, since SIM only

monitors on a limited number of ions, the sensitivity of selected ion monitoring can be dramatically increased (up to 100-fold and more), which can be utilized for quantitative measurement.

3.1.3. Sensitivity

Although a mass spectrometer is not the highest sensitive detector available for LC, a mass spectrometer gives rich information content and excellent selectivity which have compensated its sensitivity and made it the most attractive detector for liquid chromatography. Sensitivity of a mass spectrometer is affected by ionization techniques, interfacing techniques, characteristics of samples and chromatographic conditions.

Both electrospray and ionspray ionization and interfacing techniques have demonstrated the best sensitivities compared to other ionization and interfacing techniques. For both electrospray and ionspray, detection limits can be achieved in the low picomole to high femtomole ranges in the scanning mode. Selected ion monitoring of both electrospray and ionspray give even better detection limit at femtomole range. The detection limit for thermospray is generally in the low picomole. Detectors other than electrospray, ionspray and thermospray offer poorer detection limits [45,46,47].

3.1.4. The power of LC/MS

High-performance liquid chromatography has been developed into one of the premier separation techniques. One of the reasons for its explosive growth of applications is its suitability for separating nonvolatile species or thermally fragile ones. At present the mass spectrometer still is unique in that it offers sensitivity, selectivity, and near universal detection. LC/MS combines high-performance liquid chromatography (LC) with mass spectrometry (MS), which offers the possibility of taking advantage of both chromatography (LC) as a separation method and mass spectrometry (MS) as an identification as well as a quantitative method. LC/MS is an extremely powerful combination [48].

3.2. Disadvantages of LC-MS

3.2.1. LC liquid flow rate vs. MS high vacuum

One characteristic of mass spectrometer is its operation under high vacuum. Under such circumstances, collisions between ions and background molecules are avoided, which makes it possible for ions generated in the ion source can reach the detector without being hindered. For EI ionization, pressures are maintained in the source housing at range from to 5×10^{-6} torr. CI ionization allows higher pressures in the source housing. Typical working pressures in a CI source housing ranges from 1 to 5×10^{-4}. The working pressure in the source housing which surrounds the ion source is lower than one in the ion source.

The maximum pressure in the ion source housing given by a conventional pumping system is about 2.5×10^{-4} torr. The pressure corresponds to the vapor flow rate of 5-7.5 cm^3/min. This figure represents a vapor flow rate that can be allowed to enter the source housing under a conventional pumping system. A flow of 5 μl/min water can be vaporized to generate a vapor flow of 6.25 cm^3/min. A flow of 15 μl/min of acetonitrile can generate a similar vapor flow when it is completely vaporized. However, a conventional LC column has approximately 1 ml/min solvent flow rate, which is a much higher flow rate than the rate that can be admitted by the mass spectrometer ion source under normal circumstances [49].

There are a number of possible solution to solve the incompatibility of liquid flow rate and MS high vacuum. (1). The pumping capacity can be enlarged by choosing different pumping system or by the addition of a cryopump. The maximum working pressure of 50 μl/min can be given by a cryopump [50]. (2). Flow rate can be reduced by using a mechanical device to remove solvents prior to introduction of sample into the ion source. The moving-belt interface was designed for this approach [51,52]. (3). Liquid flow splitting is another approach to solve the problem of incompatibility. The effluent from a conventional LC column can be split and only a small part of the effluent is introduced into the CI ion source of a mass spectrometer. This approach wastes sample

and is not acceptable when the sample quantity is limited [53]. (4). The effluent splitting can be replaced by using miniaturized LC columns. Narrow-bore, packed-capillary and open-tubular columns can be used to eliminate the flow rate [54,55]. (5). Recently, atmospheric-pressure ion sources and ionization such as electrospray and ionspray ionization and interfaces are introduced [56,57].

3.2.2. Volatilization of the sample

Both electron impact and chemical ionization techniques are conventional ionization techniques. Samples in both EI and CI ionization techniques must be thermally volatilized prior to ionization. For many large molecules and thermal fragile compounds, this prerequisite of volatilization is unacceptable. In order to overcome this problem, soft ionization techniques such as fast atom bombardment, thermospray and electrospray ionization have been introduced.

3.2.3. Incompatibility with nonvolatile LC mobile phase

In the application of liquid chromatography, non volatile composition of the mobile phase is often used. The conventional ionization techniques such as EI and CI can not afford the large amount of non volatile mobile phase. The nonvolatile mobile phase of liquid chromatography is incompatible with the prerequisite of volatilization by a mass spectrometer. In that respect, only volatile buffers such as ammonium acetate, acetic and trifluoroacetic acid should be used [58,59].

4. LC/MS interface designs

There are many different approaches of interfacing LC and MS. Originally the design of an interface between LC and MS was focused on devices and methods that overcome the incompatibility of the liquid flow rate and the mass spectrometer's high vacuum. More recently, attention has been focused on the practical use of ionization techniques that do not require sample volatilization.

There have been a number of successful approaches to LC/MS interface designs. These range from mechanical transport of solute to the mass spectrometer after solvent removal external to the vacuum system (molving-belt interface) to bulk solution introduction with or without splitting (direct liquid introduction interface) to direct ionization methods involving the entire solvent stream (thermospray, electrospray, and ionspray interfaces). These methods will be discussed in detail in the following sections.

4.1. Transport interfaces

4.1.1. The basic principles

Interfacing the effluent from a liquid chromatograph to a mass spectrometer is more difficult than for GC. A number of devices have been proposed to eliminate the carrier liquid before the vaporization of sample constituents. In a transport interface, the effluent containing sample from the LC is carried by mechanical means from the exit of the LC column to the MS ion source, while the mobile phase is removed. This principle was the basis of the first commercial LC/MS interface and is still used in current transport interface. In the early development [60], the moving-belt interface included five successive steps (Figure 4.1.1): (1) The effluent was deposited on a continuously moving polyimide belt; (2) The belt passed under an optional infrared heat source to evaporate the solvent; (3) The belt then passed through two successive vacuum chambers where solvent was evaporated under reduced pressure and was pumped off; (4) Flash vaporization (desorption) of the analyte was effected as the belt passes through a heated chamber adjacent to the source; (5) On the return path the belt passed over a clean-up

heater to remove residual solvent and sample and finally through a wash bath (scrubber) to remove nonvolatile materials, thus preparing the belt to receive the next portion of effluent [61,62].

4.1.2. The modern designs

In the early designs, the column liquid effluent was directly deposited on the belt surface via a microbore column. Direct solvent deposition works well for relatively non-polar eluents at flow-rates below 1 ml/min. For applications with higher flow-rates and more polar mobile phase compositions some optimization of the solvent deposition step is necessary. With polar eluents the degree of wetting will affect the uniformity of the layer after evaporation. Droplet formation on the surface causes an uneven surface coverage of analyte on the belt which leads to distorted peak shapes. A high solvent flow rate and a less volatile polar mobile phase (used in reversed-phase LC) result incomplete evaporation which will allow the mobile phase to reach the first vacuum lock. This will significantly increase background noise. These problems can be solved by the application of nebulization techniques [63,64,65].

The use of a pneumatic nebulizer with a preheated gas provides a fine spray that is uniformly coated onto the belt, and at the same time a large fraction of the solvent is evaporated during spraying (so that infrared heating is no longer needed) have all improved compatibility with aqueous (polar) phases, and high liquid flow. A schematic diagram of the heated gas pneumatic nebulizer developed by Smith and Johnson is shown in Figure 4.1.2. This aerosol liquid deposition device consists of a concentric pneumatic nebulizer in which preheated argon is used to aid the evaporation of higher mobile phase flow rates or less volatile solvents.Preheating of the inert gas allows solvents of intermediate volatility (such as 2-propanol) to be evaporated without additional heating. For high solvent flows, or solvents of extremely low volatility (e.g., water), strip heaters located just above and below the moving belt aid in solvent evaporation. In addition to

the ability to handle enhanced solvent flows, analyses using this deposition system have demonstrated improved peak shapes.

However, even with a pneumatic nebulizer, water contents exceeding 50% at flow-rates higher than 0.5 ml/min are still problematic. More recently, thermospray nebulization has proved to be a very effective replacement for pneumatic nebulization, especially when solvents with high water content and/or high solvent flow rates are used. The advantage of this approach is that most of the solvent (~ 95%) is removed before sample deposition onto the belt. This eliminates the need for strip heaters and extra differential pumping stages. One thermospray solvent deposition device is illustrated in Figure 4.1.3. The thermospray solvent deposition devices were reported by various group [66,67,68]. The thermosprayer consists of a 0.15 mm i.d. stainless steel capillary tube brazed into a copper block heated by tow cartridge heaters. The thermosprayer is positioned about 1 cm above the belt and approximately 220-240°C is necessary to produce a fine mist covering the belt. The thermospray temperature is used to adjust the degree of solvent vaporization, and the fineness of a mist. In addition to modifications on solvent deposition, passing the belt directly into the source has also improved sensitivity and decreased the sample heating requirements [69].

4.1.3. The advantages of MBI

The moving-belt interface has two particular advantages. The principal advantage is that since the mobile phase has been evaporated by the time the sample reaches the ion source, the belt interface is compatible with a variety of ionization modes including electron impact (EI), chemical ionization (CI), and fast atom bombardment (FAB). The type of ionization is not restricted to CI-like conditions, so that a comparison of data with the extensive collections of EI reference spectra may be made. The second advantage is that use of the moving-belt system places few restrictions on the LC system. In particular, since buffers can be washed from the belt after it has left the ion source, nonvolatile buffers can be used. This is not the case with most other LC/MS interfaces.

4.1.4. The disadvantages of MBI

There are a number of practical problems associated with the belt interface in addition to its relative expense and complexity. One problem is a gradual build-up of background and carryover from the belt during use since it is particularly difficult to ensure efficient removal of nonvolatile residues from solvent and sample before recycling. The high belt background problem has been noted at low mass [62]. Since high-performance liquid chromatography (HPLC) is normally used with higher-molecular-mass compounds, this often is not a severe problem. Carry-over can, of course, be alleviated or removed by optimizing the scrubber process. The second problem is more fundamental. It is that nonvolatile or thermally labile samples cannot be analyzed, at least not by evaporation from the belt. Under these conditions, such compounds may give spectra only at very high microgram sample levels but most often give no useful data at all [70].

The combination of the moving belt with FAB ionization was proposed as a promising way of extending the application of the transport interface to the analysis of more polar and thermally labile compounds [71,72,73]. Although experimenters initially added a matrix compound such as glycerol through a tee at the end of the column, it was later decided that a matrix compound was not essential since the movement of the belt continuously offered a fresh sample surface for bombardment. Under these conditions a number of authors were able to demonstrate some success in the LC/MS analysis of peptides and oligosacchrides in the molecular weight range 1000-4000. However, the method has never really reached the stage of routine applicability and has, to a great extent, been superseded by continuous flow-FAB.

4.2. Direct liquid introduction

4.2.1. Diaphragm based DLI interfaces

In direct liquid introduction interface (DLI), the LC column effluent is introduced directly into the mass spectrometer through some form of restriction. In commercial

systems, and in most laboratory-built interfaces as well, a laser-drilled orifice (2-5 μm in diameter) in a replaceable diaphragm (Fig. 4.2.1) is used as a restrictor [74,75]. So long as there is an adequate flow through this orifice, a liquid jet forms and then immediately breaks into small droplets. Nickel or stainless-steel diaphragms are mostly used. The probe of the interface is cooled by circulating cooling water prior to reaching the orifice in order to prevent evaporation of the liquid inside the probe. This jet then passes into a heated desolvation chamber where the droplets are vaporized, and the solvent vapor and sample enter the ion source of the mass spectrometer.

Since the small diaphragms often become plugged, research has been directed towards other restrictions giving better reliability and lower costs in routine applications. Diaphragms with pinhole diameters up to 10 μm ID have been used [76]. The minimum flow-rate to form a liquid jet from such a pinhole is 30 μl/min (The minimum liquid jet flow-rate for a 4 μm ID pinhole is 7 μm/min.). The higher liquid flow-rates can be utilized when the vacuum system is modified to evacuate the ion source directly with a mechanical pump.

4.2.2. Other DLI interfaces

Replacement of diaphragms with short narrow bore capillary tubes, which are much cheaper than the precision pinholes used as diaphragm, has also been tried [76]. The same liquid flow-rates are necessary to form a liquid jet at a capillary tube as at a diaphragm. However, the pressure drop over a capillary tube is considerably higher than that over a diaphragm. As the pressure drop over a capillary tube is proportional to the solvent viscosity, which is strongly temperature dependent, warming the liquid by means of recirculating hot water through the interface probe can be used to overcome the problems with this higher pressure drop. This approach is called "hot-DLI".

The mass spectrometer vacuum system can tolerate only a limited solvent flow if suitable operating pressures are to be maintained in the source housing and in the

analyzer. DLI interfaces designed for use with conventional LC columns (approximately 1 ml/min solvent flow rate) incorporate a flow splitter, usually a needle valve located downstream from the orifice. This allows 5-15 μL/min of the solvent flow to enter the ion source and provides the back-pressure necessary for jet formation. If cryopumping or larger pumps are employed, flow rates up to 20-50 μL/min can be accommodated.

The permissible flow rates for DLI are, however, more compatible with micro-LC column operation. For example, flow rates with 1 mm i.d. columns are in the range 10-50 μL/min so that no split is necessary. Under these circumstances, all of the sample may be introduced into the ion source with obvious advantages where only a limited amount of sample is available [77].

Another direct liquid introduction with a microcolumn has been reported [78]. A microcolumn, which may be an open-tubular or a packed capillary column, is drawn to a fine tip at the exit and then introduced directly into the ion source. When the column tip is heated as well as tapered, vaporization occurs at the tip rather than inside the column resulting in much better control of both solute and solvent evaporation. The solvent flow through these open-tubular columns is so low (30 nL/min) that electron impact or chemical ionization (with the addition of reagent gas) spectra may be obtained. However considerable thermal degradation was observed with various compounds.

4.2.3. Desolvation chamber

The desolvation chamber is interfaced to a conventional chemical ionization ion source [79,80,81]. The purpose of inserting a desolvation chamber is to provide a heated zone between the probe tip and the ion source in order to promote the desolvation of the droplets. From the desolvation chamber a mixture of solvent vapors, desolvated analytes and residual tiny droplets enter the ion source. The vapors of the mobile phase solvents act as a reagent gas in chemical ionization(CI). Therefore, this type of CI is designated as

solvent-mediated CI. The nature of the chemical ionization reactions depends on the composition of the reagent gas.

4.2.4. Solvent mediated chemical ionization

In most DLI experiments a mixture of water and a polar organic solvent, e.g., methanol, is used. Typical reagent gas spectra of water-methanol mixtures comprise protonated methanol, as well as methanol and methanol-water clusters. In a water-methanol mixture protonated methanol (clusters) is the most abundant species over a rather wide range of solvent compositions, which is the result of the higher proton affinity of methanol (777 kj/more for methanol vs 727 kj/mole for water). The relative abundances of the various ions in the reagent gas spectrum depend on ion source temperature, pressure, and to some extent on the solvent composition as well. At higher pressures the abundance of the cluster ions increases in favor of the low molecular weight species [82].

The ion source pressure can exert a distinct influence on the fragmentation depending on the nature of the solute, as is common in CI. The influence of the ion source temperature is more complicated, because it appears to be difficult to discriminate between the temperature effects of the ion source and of the desolvation chamber. At low temperatures the desolvation process will not be completed, resulting in $[M+H+solvent]^+$ adduct ions and other solute-solvent cluster ions. At higher temperatures these clusters normally disappear. However, with particular solutes and solvents these adduct ions are part of the "normal" CI spectrum. e.g., when the proton affinities of solvent and solute are similar. At higher temperatures the solvent-cluster ions decompose, actually resulting in a change in the reagent gas composition. The protonated molecules and protonated clusters differ in proton affinity, as well as in reactivity. In addition, with more volatile solutes the increased vaporization of the solute at higher temperature will enhance the abundance of the solute ion. Besides these effects, the temperature also influences the fragmentation pattern either by increasing the fragmentation of the ions produced or by protonation of the thermal decomposition products [83,84].

The high source pressures encounted in DLI yield CI spectra, both positive and negative ion, with the solvent vapor acting as the reagent gas. The character of the chemical ionization spectra may be changed by the use of additives introduced directly into the ion source in both the positive and negative ion modes [85].

The DLI interface can be used with most common LC solvents and with volatile buffer materials such as ammonium acetate, ammonium formate, ammonium hydroxide, and acetic and trifluoacetic acids. The continued use of highly aqueous phases may, however, lead to a reduced lifetime for the ion source filament. A particular practical difficulty with the DLI interface is the very small size of the orifice, which makes it very susceptible to plugging by particulate matter. Location of the orifice in a replaceable diaphragm gives a considerable advantage in this case. In addition, the use of the DLI interface has been restricted to the analysis of more volatile samples.

4.3. Thermospray

4.3.1. The TSP interface

The term thermospray has been applied both to a method of introducing high flow rates of liquid into a mass spectrometer vacuum system and to a method of ionization. In a modern TSP interface [86,87,88,89,90], a flow of 1-1.5 ml/min solvent is rapidly vaporized in a heat-resistive metal capillary tube with an inside diameter of approximately 100 μm. The signal from a thermocouple is fed back to the heating circuit to maintain the situation where a constant high percentage of the liquid flow is volatilized. Under these circumstances the vapor that expands from the end of the capillary has sufficient energy to transform the remainder of the liquid flow into a mist of fine droplets (Figure 4.3.1).

The jet that carries the droplets expands into the ion source where additional heat is added to complete the vaporization process. Independent heaters surrounding the capillary tip and the main ion source block allow the optimization of desolvation conditions and prevent sample condensation. The process by which sample ions then

originate from these droplets in the presence of a volatile electrolyte (thermospray ionization) has been described before. Most sources now also include a discharge electrode or filament to assist in ion production [92]. In the absence of a volatile electrolyte, if this electrode is held at a suitable voltage relative to the ion source block, a discharge ensures that ionizes the solvent vapor and hence the sample by a chemical-ionization process.

The other important feature of the inlet system is the rotary pump attached to the ion source. This pump removes the great majority of the solvent vapor that enters the ion source (and certainly some of the sample) while only a small fraction enters the source housing via the orifice in the ion exit plate. It is the use of this pump, which may be supplemented by a cold trap, that allows the introduction of such high liquid flow rates directly into the ion source region.

The original thrmospray interface offered no form of ionization other than the interaction of a volatile electrolyte with the sample and, as a consequence, was restricted to the analysis of samples in solutions with a relatively high water content. The subsequent addition of a filament and discharge electrode has greatly increased the versatility of the system so that samples in mainly organic solvents and solutions that do not contain a volatile buffer may also be analyzed. Another advantage of discharge ionization is that, since its performance is much less dependent on the nature of the solvent, it is generally more suitable than thermospray ionization in LC/MS experiments, where a gradient that covers a wide range of solvent compositions is used. Most thermospray ion sources also include an ion repeller electrode which can be used to improve sensitivity, particularly for higher mass compounds, as well as to induce controlled fragmentation of quasi-molecular ions in some cases [93,94,95,96].

4.3.2. The advantages of TSP

Although thermospray comprises a special inlet system and a special ion source, and although thermospray ionization is another relatively mild process that often fails to

give any fragment ions, it is, nevertheless, a very popular LC/MS technique in use today. This is because it is directly compatible with conventional LC flow rates (1-2 ml/min), can deal (especially in conjunction with discharge ionization) with any solvent, and is applicable to samples that cover a very wide range of volatility. Thus, thermospray will deal with a wider range of LC analyses under conventional LC conditions than any other interfacing technique currently available.

4.3.3. The disadvantages of TSP

Thermospray does have its disadvantages. For example, nonvolatile buffers cannot be used with thermospray. Again, thermospray ionization is a relatively mild ionization technique and many compounds show only quasi-molecular ion species, although the use of MS/MS techniques in conjunction with thermospray LC/MS has proved quite useful here. The operating temperatures for optimum sensitivity depend critically on the sample to be analyzed and on the solvent system used. In this respect, it is found that the discharge ionization mode requires less critical setting of operating temperatures.

4.3.4. The optimum of the TSP

Many experimental parameters, such as solvent composition, flow-rate, vaporizer temperature, repeller potential and ion source temperature have great influence on the performance of the TSP interface and ionization [97,98,99]. First, attention is focused on TSP buffer ionization.

In TSP ionization a volatile electrolyte must be present in the mobile phase or added post-column [100]. Among the varieties of electrolytes, ammonium acetate is the most widely used electrolyte. Ammonium formate is applied for the negative ion TSP ionization [101]. For the application of reversed-phase LC, methanol and acetonitrile are most popular organic modifier. The percentage of the modifier content of the mobile phase should not exceed 50% because a high water content is often favorable for TSP

ionization. When the solvent used has higher polarity, less modifier contents can be used without deteriorating the separation. At present no good alternatives for methanol and acetonitrile have been found [102]. The external ionization such as filament-on or discharge-on should be applied at high modifier contents. Since TSP buffer ionization is similar to CI ionization, the modifier (in the negative ion mode) can be selected according to relative proton affinities of solvent constituents and analytes.

In most commercial TSP interface, flow-rates of 1.0-2.0 ml/min are commonly used. The flow-rate higher than 1.0-2.0 ml/min will cause vacuum problems in the MS manifold, and the flow-rate lower than 1.0-2.0 ml/min is also not applicable in TSP buffer ionization with the commercial vaporizers because the internal diameters are too large. In general the low flow-rate is preferred.

The vaporizer temperature that must be used to achieve the optimum nebulization of the liquid is determined by the solvent composition and flow-rate. Nearly complete vaporization is needed for successful TSP buffer ionization [87]. The degree of vaporization is of utmost importance. Careful adjustment of the vaporizer temperature for a particular solvent composition and flow-rate is necessary to avoid signal instabilities (too high vaporizer temperatures). The vaporizer exit temperature is typically 200 °C, while the temperature near the beginning of the vaporizer capillary is 100°C. It should be noted that with the fixed vaporizer the exit temperature is monitored and reported, while with the flexible vaporizers the temperature near the beginning is monitored and reported. The setting of the vaporizer temperature is especially critical with thermolabile compounds; the decomposition products can be ionized as well and may give peaks in the mass spectrum that are difficult to explain. Lowering the source block temperature may be beneficial for thermolabile compounds as well. It should be mentioned that the vaporizer temperature depends on the conditions (age, contamination, etc.) of the vaporizer; the optimum vaporizer temperature may vary from day to day and for a particular application may deviate significantly from values reported in literature.

The repeller potential has a distinct influence on the TSP buffer ionization. For simple analytes the ionization is most likely based on gas-phase ion-molecule reactions. In general, no signals are observed at higher effective repeller potentials indicating that the ammonium cluster ions play a more important role in the gas-phase ionization than the protonated acetic acid and water formed at higher repeller potentials [103]. For ionic compounds, such as peptides, the repeller potential may influence the ion evaporation efficiency [104].

With the external ionization modes, i.e., filament-on and discharge-on, generally more degrees of freedom are available. Although ammonium acetate is still used quite often with the external ionization modes, the applicability range can be enlarged when the buffer is left out. With the external ionization mode the ionization takes place by gas-phase ion-molecule reactions. Without the ammonium acetate in the mobile phase a wider range of analytes can be ionized. The solvent can be selected relatively freely; the selection must be based on the relative proton affinities of the solvent constituents and of the analytes. Solvent adduct ions or solvated protonated molecules may be observed as well. The combination of flow-rate and vaporizer temperature is less critical, as long as the analyte is not thermolabile. The repeller potential can be used to change the ionization conditions in the source [95,96]. Collisional-induced dissociation of the protonated molecules is also present at higher repeller potentials.

4.4. Continuous flow, fast atom bombardment (CF-FAB)

4.4.1. Introduction

The most import aim in developing LC-MS is extending the analytical capabilities with respect to the amenable polarity range of compounds. In this respect the ionization of the analytes plays a dominant role in the overall performance of any system developed. As a consequence, the incorporation of soft ionization techniques in LC-MS coupling is of major interest. One of the most applied soft ionization techniques nowadays is fast atom bombardment (FAB)[105,106]. Continuous flow, fast atom

bombardment (CF-FAB) is an important interfacing technique which, after thermospray, is the most widely used method. It is also sometimes known as dynamic FAB[107]. It is another form of direct liquid introduction, but one that has been developed almost totally for use with FAB ion sources.

4.4.2. CF-FAB interfaces

The technical principle of a CF-FAB interface (Figure 4.4.1) is very straightforward. A liquid flow, typically 5-10 μL/min with standard pumping systems, enters a fast atom bombardment ion source through a narrow fused silica capillary (50-75 μm i.d.). This capillary is generally contained within a probe that can be brought up to or removed from the ion source by means of a standard vacuum lock. Flow splitting is required unless some form of microcolumn is used. Nonvolatile buffers are not recommended with CF-FAB.

A small percentage of a FAB matrix material, such as glycerol, is added to the liquid flow either at the column exit or, in some cases, directly to the aqueous component of the chromatographic mobile phase. The liquid emerging from the capillary forms a target on the metal end of the probe, which is bombarded by the atom or ion beam as in a conventional FAB source. Some heat is applied to the probe tip solely to prevent freezing as the liquid evaporates in the mass spectrometer vacuum. Post-column addition is another way to add the FAB matrix. Both capillary LC and OTLC columns adapted to CF-FAB by using a coaxial column in which the LC column is inserted within the column carrying the FAB matrix have been described [109,110].

For the optimum use of CF-FAB as an LC/MS interface, two conditions should be met: (1) The liquid flow should form a stable, continuous film on the probe tip in which the previously eluted sample is removed from the area where fast-atom bombardment takes place; and (2) the liquid flow rate should be equal to the liquid evaporation rate. Probably the most satisfactory method of accomplishing this is to interpose a small metallic frit between the end of the capillary and the mass spectrometer vacuum. Not

only is the metal surface of the frit easily wetted but also the improved thermal conductivity of the metal surface and the narrow orifices in the frit encourage a very stable liquid evaporation process. A further advantage of the frit construction is that the glycerol concentration required can be reduced from approximately 5% to 0.5%.

4.4.3. The advantages of CF-FAB

The range of compounds that may be analyzed by CF-FAB is the same as for conventional FAB and includes thermolabile and ionic materials as well as nonvolatile biopolymers such as peptides, oligosaccharides, and oligonucleotides. In addition, CF-FAB has some advantages compared with conventional FAB. For example, suppression of the ion current due to one sample in the presence of another sample is much less evident in the continuous flow technique; and chemical background signals due to the matrix are reduced with correspondingly improved detection limits [109].

4.4.4. CF-FAB operating parameters

The production of a stable and thin liquid film is of utmost importance in CF-FAB operation; it depends on many experimental parameters. The most important ones are the mobile phase composition, the flow-rate, the material, shape, position and chemical treatment of the target, the pumping capacity and the temperature of the ion source and the relative position of the capillry tip to the target surface.

A filter pad (or wick) at the bottom of the ion source can improve the stability in CF-FAB operation [111]. The filter pad absorbs the remainder of the effluent that is not yet evaporated and flows from the target, and provides for a smooth evaporation of that liquid. However, the non-volatile constituents are concentrated in the wick., and therefore, an exchange of the filter pad is needed after a certain time.

Several parameters influencing the wettability of the target have been studied in the LC-MS mode [111]. In a comparative study of various target materials, i.e., stainless steel, copper, nickel and silver, it appeared that the latter three materials yield a stable

background within a few minutes, while it takes about 15 minutes for stainless-steel. This difference is attributed to the good thermal conductivity of the latter three metals. However, the stability observed with the nickel target lasts for a short period only, while with the copper and silver targets cluster ions are observed. Furthermore, the roughness and consequently the wettability of the target materials can be improved by means of a treatment of the surface with concentrated hydrochloric acid (for a stainless steel target) or nitric acid (for a silver or a nickel target). The improved wettability lasts for a whole working day in the case of the stainless steel target, but for only two hours in the case of the nickel target, while it is not applicable to the silver target. As a result, a stainless steel target combined with chemical roughening and a wick appears to be most favorable. Recently, an improved stainless-steel target with a gold plated drain channel has been described. This target yields almost instantaneous stability and can be used at higher flow-rates of typically 15 μl/min [112].

The mobile phase composition obviously is an important parameter in achieving stable liquid film conditions as well. The glycerol content has to be optimized depending on the flow-rate in order to provide the optimal glycerol supply rate to the target surface.

The ion source temperature also affects the evaporation rate of the mobile phase components. As a result, changing the flow-rate or the water content of the mobile phase requires optimization of the source temperature in order to prevent freezing of the solvents on the target. Generally, ion source temperatures of 40 - 80 °C are used for mobile phases containing a low and high water content, respectively.

In conclusion, it can be stated that tuning of the CF-FAB conditions needs a multiparameter optimization approach in which the most important parameters for a given target, ion source design and pumping capacity are the flow-rate, the glycerol content and the composition of the mobile phase and the ion source temperature.

4.4.5. Other approaches to CF-FAB

For quadrupole instruments, a commercial interface design, consisting of a capillary tube that contacts a standard FAB probe near the ion extraction assembly, has been improved by applying an evaporation device. This system is based on sustaining the liquid transport across the probe surface by channeling the liquid onto a high capacity absorbent. Sufficient heat could be provided to bring the flow system close to a state of a mass-transfer dynamic equilibrium [113].

4.5. Electrospray and Ionspray

4.5.1. The electrospray interface

As mentioned in the previously chapter, in the electrospray ionization process, a flow of sample solution is pumped through a hypodermic needle held at a potential of a few kilovolts relative to an opposing metal plate. Charging of the liquid occurs and it emerges from the needle as a mist of very fine, charged droplets. This spraying process takes place in the atmosphere [114,115,116]. A typical ESP interface for LC-MS was developed by Whitehouse et al, which is shown in Figure 4.5.1 [117]. Sample solutions enter into a spray chamber by pumping through a stainless-steel hypodermic needle at a flow-rate of 5-20 µl/min. The needle is kept at ground potential. The cylindrical electrode is set at -3.5 kV for positive ion detection. The metallized inlet and outlet ends of the glass capillary are set at -4.5 kV and +40 V, respectively. For negative ion detection the polarities of the various potentials are reversed. The liquid is electrosprayed from the tip of the hypodermic needle and the droplets formed are further dispersed by means of a countercurrent stream of heated nitrogen gas with a flow-rate of 150 ml/min. The solvent vapor from the rapidly evaporating droplets is swept away by the bath gas, while the ions formed, that are coming near the inlet of the glass capillary, are entrained in dry bath gas and transported into the first vacuum chamber, forming a supersonic beam. Ions at supersonic speeds easily overcome the potential barrier in the glass capillary. The first vacuum chamber is evacuated down to 0.05 Pa by means of a

$1 m^3/s$ (10^6 ml/s) diffusion pump. The core of the supersonic jet is sampled by a 2 mm ID skimmer, kept at -20 V, and transported into the quadrupole analyzer region for mass analysis.

The electrospray LC-MS interface looks promising [118,119,120] since the countercurrent bath gas prevents the introduction of non-volatile contaminants into the high-vacuum region, the ionization efficiency appears to be relatively independent of the molecular weight of the analyte, and the system is very stable, neither very dependent on small liquid flow-rate fluctuations nor on modest changes in temperature and flow of the bath gas. The major drawback of the ESP interface in LC-MS application lies in the fact the the maximum allowable flow-rate is limited to 10 µl/min; the lower flow-rate the better the performance.

Various modified ESP interfaces have been described [121,122]. The ESP interface has become commercially available from several instrument manufacturers. Generally, an ESP mass spectrometer is equipped with three pumping stages, i.e., the first between the ion-source sampling nozzle and the (first) skimmer, the second between the skimmer and the mass analyzer entrance opening or slit, and the third at the mass analyzer.

4.5.2. ESP interfaces for a quadrupole MS

An ESP interface for a quadrupole MS was described by the group of Chait [123]. The main difference from previous designs is in the combination of the techniques used in transportation and desolvation of the ion-solvent clusters. Desolvation is effected by means of controlled heat transfer to the clusters in the 203 mm x 0.5 mm ID stainless-steel transfer capillary and by means of collision activation in the low-pressure (150 Pa) region between the capillary exit and the skimmer. Data are presented for various peptides and proteins in the molecular-weight range of 5-77 kDa. Flow-rates in the range of 0.5-2 µl/min are applied.

4.5.3. ESP interfaces for a magnetic sector MS

ESP interfaces for magnetic sector instruments have also been described by various authors [124,125,126]. The major difficulties in coupling an atmospheric-pressure ion source to a sector instrument are related to the large pressure difference between ion source and analyzer, which requires extensive pumping in order to avoid discharges and electrical breakdown in the instrument as well as extensive collision activation of the ions. In general, an additional pumping stage is used, i.e., a total number of four stages instead of three. Additional precautions for electrical breakdown are sometimes incorporated.

4.5.4. The ionspray interface

The ionspray (ISP) interface originally was introduced in order to combine the principles of ion evaporation and electrospray [114,127]. The main advantage of the ISP interface over the ESP interface is the higher liquid flow-rates, that can be accommodated by ISP. Flow-rates as high as 200 μl/min have been reported [128]. The higher flow-rates are possible as a result of the combined action of electrospray and pneumatic nebulization.

A schematic diagram of the ISP interface is shown in Figure 4.5.2. It can be characterized as a nebulizer-assisted ESP interface [128]. The column effluent flows through a 50 μm ID fused-silica (or stainless-steel) capillary, which fits inside a 200 μm ID stainless-steel capillary kept at -3 kV (for negative ion detection). A 0.8 mm ID PFTE tube with a narrower PFTE insert at the tip surrounds the stainless-steel capillary, and nitrogen gas flows through the PFTE tube. The dimensions of the PFTE insert are chosen to provide for linear gas velocities exceeding 200m/s at the tip, which is needed for successful pneumatic nebulization. The relative position of the three concentric tubes must be adjusted to give a fine symmetric spray plume. In practice, the fused-silica capillary protrudes from the stainless steel capillary, which also protrudes from the PFTE tube.

The ISP interface provides for a mist of fine droplets that evaporate in an atmospheric-pressure ion source. Ion-solvent clusters are sampled to the high-vacuum analyzer region of a (triple) quadrupole instrument through a special skimmer. An important feature of the sampling device is the nitrogen curtain gas, which acts as contamination prevention for the skimmer, but also induces solvent evaporation from the droplets and declustering by means of collision-induced dissociation of ion-solvent clusters entering the skimmer. The interface is positioned 5-10 mm off-axis of the skimmer; on-axis operation gives intense solvent cluster ions and a suppression of the analyte signal.

Typical ISP mass spectra contain intense protonated molecules without any fragmentation. In some cases some solvent cluster and/or adduct ions are observed. The system has good sensitivity and stability. Unlike ESP, the ISP system can also handle a mobile phase with high water content and is easily applicable in gradient elution. Although flow-rates up to 200 μl/min can be accommodated, the system shows better sensitivity at lower flow-rates. The ion spray system also shows improved performance relative to the TSP, since ISP operates at room temperature, while heating is applied in TSP.

5. Application of LC-MS in analysis of amino acids and peptides

In the analysis of amino acids, peptides and proteins the progress in LC-MS interfacing has been dramatic. The interface designs discussed above such as MBI, DLI, TSP, CF-FAB, ESP and ISP have all been applied to the analysis of amino acids and peptides. This development is outlined below. Since molecular weight determination and sequencing of peptides and proteins are extremely helpful in characterizing synthetic peptides and recombinant products as well as in elucidating their structures, the attention of this section is focused on the ability to obtain molecular weigh and sequence information from the different interfacing techniques for LC-MS.

5.1. Applications using MBI

In the analysis of amino acids and peptides, one of the earliest types of interfaces with respect to the choice of the ionization methods was the moving belt. A moving-belt LC-MS ammonia CI mass spectrum of the underivatized cyclic peptide cyclosporin A (molecular weight 1201) was reported by Van der Greef et al.[129], which shows the ability of the moving-belt system to handle high-molecular-weight polar compounds. However the moving-belt interface seems to be the least sensitive, and also appears to induce thermal decomposition at low levels [130]. In addition, even if a spectrum can be readily obtained, its interpretation can sometimes be difficult. The permethylation derivatization has improved the sensitivity (100 μg N-acetyl permethylated Leu-enkephalin) and at the same time provided readily interpretable spectra [131,132]. N-permethylated derivatives generally offer two major advantages: a) volatility is increased by permethylation. The presence of -CO-NH- groups in peptides causes hydrogen bonding which leads to low volatility. Permethylation of the -CO-NH- by replacing the hydrogen with $-CH_3$ blocks the hydrogen bonding; and b) it becomes apparent that mass spectral fragmentation is in all cases extremely simplified as a result of the methylation.

The mass spectra consist almost exclusively of the "sequence-determining" peaks which result from CO-NCH_3 cleavages [133,134,135]. The analysis of N-acetyl-N,O,S-permethylated oligopeptide derivatives using an MBI operated in an isobutane-CI was reported by Yu et al. [131,132]. In their report a heated pneumatic nebulizer was employed to provide a fine spray for sample deposition. This application of a heated gas pneumatic nebulizer improved compatibility with aqueous phases and high liquid flow, which permitted the effective use of a water-methanol gradient covering the range from 5% to 95% water at mobile phase flow-rates of 0.5 ml/min in reversed-phase liquid chromatography (RPLC). The largest intact peptide that was demonstrated to be amenable to this technique was the N-acetyl-N,O,S-permethylated Met-enkephalin (Figure 5.1.1) (molecular weight 713).

Tyr-Gly-Gly-Phe-Met

Ac-Me-Tyr-Me-Gly-Me-Gly-Me-Phe-Me-Met-O-Me

(Figure 5.1.1. N-acetyl-N,O,S-permethylated Met-enkephalin, MW 713)

A HPLC-MS total-ion chromatogram of Met-enk following permethylation is shown in Figure 5.1.2. The major peak (B) in Figure 5.1.2 is identified from its mass spectrum as N-acetyl-permethylated Met-enk (Figure 5.1.3a). The two smaller peaks are peak (A) identified as the N-acetyl-permethylated tetrapeptide Tyr-Gly-Gly-Phe which is produced by hydrolysis of Met-enk during the derivatization process (Figure 5.1.3b) and peak (B) identified as C-methylated Met-enk (Figure 5.1.3c). C-methylation is a by-product in permethylation of peptides and gradient elution HPLC can separate the C-methylated analogues from the N-acetyl-permethylated derivatives [136].

The fragment ions in those CI spectra (Figure 5.1.3a,3b,3c) from the three major peaks shown in Figure 5.1.2 are easily identified for the permethylated derivatives of the respective oligopeptides. Figure 5.1.4 illustrates how these fragment ions are related to the N-acetyl-permethylated peptides including the C-methylated derivative.

Possible sequencing of the octapeptide Phe-Val-Glu-Trp-Leu-Met-Asn-Thr derived from the C-terminus of glucagon is achieved by using enzymatic hydrolysates (Phe-Val-Glu-Trp and Leu-Met-Asn-Thr) followed by permethylation and overlap information from the mass spectra. All these results demonstrate the potential of HPLC-MS, using a moving belt interface, for analysis of peptides following N-acetyl permethylation or from hydrolysates followed by N-acetyl permethylation. The peptide sequence can be determined by measuring the mass differences between the major peaks and using "overlap" information from the spectrum.

5.2. DLI in analyzing of peptides

Although in-source sensitivity can be achieved at ng level, DLI effluents are usually split prior to analysis, which decreases the on-column sensitivity by several orders of magnitude (The LOD was at 10μg). The potential of DLI in analyzing peptides was explored by Milon and Bur [137]. Milon and Bur studied a series of underivatized dipeptides containing proline as either the N- or C- terminal amino acid, and a series of underivatized polypeptides containing two to six glycines . Different types and position (at N- or C- terminal) of the second amino acid, the effects of positive and negative CI ionization and the pattern of fragments were examined. The molecular weight information given by the $(M + H)^+$ and $(M + H - H_2O)^+$ was available in all the dipeptide spectra, but the characteristic fragments that would lead to sequencing were not always observed and predicted.

Another problem that the DLI has is its variable tuning. In the investigation of the $(Gly)_n$ Milon and Bur used a constant-tuning procedure for optimum sensitivity of one of the compounds of interest. Under these conditions, $(M+H)^+$ ions were observed for only n=1-4 and no molecular ion species was seen for n=5 and 6. That appropriate conditions can be found for n=5 and 6 is evidenced by the observation of $(M+H)^+$ ions for Leu- and Met-enkephalins and α-amanites. When dealing with an unknown, however, the analyst often does not have an appropriate compound to tune on and, in complex mixtures, tuning

on one component may not be optimal for all components. Their conclusion was that although in all the dipeptides studied some information was available on the molecular weight, the fragmentation pattern was too unpredictable, and therefore, DLI LC-MS was not applicable for the sequencing of unknown peptides.

5.3. Use of TSP for the analysis of peptides

The use of TSP LC-MS for the analysis of peptides has proven to be quite popular. The early attempt to develop TSP by Vestal and coworkers involved detection of amino acids and peptides [138]. Most of their works on applying the thermospray LC/MS were focused on reversed-phase chromatography with ammonium acetate buffer with either methanol or acetonitrile as the organic modifier with flow rates between 1 and 2 ml/min. To the ion source there was an added a mechanical pump attached to remove excess vapor. Neither an no external filament nor a discharge electrode was needed in the ionization source to assist in ion production when the mobile phases contained significant concentration of ions in solution (10^{-4} to 1M). The limit of detection was at the subnanogram level. With weakly ionized mobile phases a discharge ionization by a conventional electron beam was utilized to ionize the solvent vapor and hence the sample by a chemical-ionization process . A mass spectrum of 6 nmole of underivatized renin substrate (Figure 5.3.1) with molecular weight of 1757 Da was analyzed. Singly, doubly and triply charged peptide peaks were detected, but no fragmentation was observed.

The potential of thermospray mass spectrometry for on-line detection of peptides separated by liquid chromatography was also demonstrated by Pilosof et al.[139]. In the course of their studies, peptides, such as the a-melanocyte stimulating hormone (α-MSH, M_r) containing 13 amino acid residues and glucagon (M_r = 3483) composed of 29 amino acid residues were among their test compounds. By use of thermospray ionization, the mass spectra obtained had abundant protonated $(M + H)^+$ and alkali-attached ions with an absence of fragmentation. The multiply charged peptide ions appeared to be dependent

on the solution pH. Figure 5.3.2 shows the mass spectrum of the α-MSH without enzymatic hydrolysis. The spectrum shown in Figure 5.3.2 does not show any singly charged molecular peaks for $(M + H)^+$, $(M + Na)^+$, and $(M + K)^+$ at m/z values of 1666, 1688, and 1704 due to the mass scan limit of the instrument at 1300. Those singly charged molecular ion peaks could not be directly detected.

Since thermospray ionization is a relatively mild ionization process, the TSP spectrum does not include sufficient fragment ions for structure elucidation. In order to increase the structural information obtained by TSP MS for peptide sequencing, immobilized enzyme columns directly coupled to the TSP system were studied [139]. More recent work has focused on direct detection of the peptides using combinations of on-line enzyme columns with an HPLC column [140]. The system uses enzymatic cleavage of proteins and liquid chromatographic (LC) separation of the components prior to thermospray MS analysis. A block diagram of a series of analytical columns connected on-line and terminating with the thermospray LC/MS is shown in Figure 5.3.3. A column containing an immobilized endopeptidase (trypsin or chymostrypsin) is used to cleave the peptide or denatured protein into primary fragments. The primary fragments are separated by a following HPLC column. A second enzyme column of immobilized exopeptidase (carboxy- or aminopeptidase) then digests each fragment as it elutes from the HPLC column. This causes the individual fragment separated by HPLC to be further digested as it emerges from the LC column and results in a set of sequence peptides. Finally, the thermospray mass spectrometer detects the peptides produced and determines their molecular weights. These peptides, which result from the loss of one, two, or more terminal amino acid residues, can all be logically related to the original fragment, and the partial sequence deduced. Through multiple combinations of endopeptidases, exopeptidases, and LC columns, it is possible to determine large portions of the protein sequence. However, protein samples at nmole level are needed for the series of experiments with the various enzymatic columns.

The combination of TSP LC-MS with either on-line or off-line enzymatic hydrolysis has a wide variety of potential applications. Many applications with this enzyme-thermospray LC/MS method have been reported by various other groups. The complete sequence of recombinant human interleukin-2 expressed in *Escherichia coli* confirmed by analyzing a trypic digest from 7 nmol of reduced carboxymethylated interleukin-2 has been reported by Blackstock et al. [141]. HPLC separations were carried out at a flow rate of 1.2 ml/min with a mobile phase contained 0.1% trifluoroacetic acid and a linear water-acetonitrile gradient. Identification of the singly, doubly or triply charged molecular ions of all tryptic fragments was made by a PC program. It is again proved that TS-LC-MS is a good rapid method for analyzing recombinant protein digests with respect to sequence confirmation.

There are a number of ways of combining the immobilized enzyme bioreactor, HPLC, and MS detection for peptide analysis. In previous examples, use of an endopeptidase bioreactor prior to HPLC separation and MS detection will enable separation of each hydrolysis fragment for identification. Furthermore, the addition of an exopeptidase bioreactor after the HPLC column will permit sequencing of the separated endopeptidase products. However, such bioreactor configurations can only be used on purified samples because no separation or column cleanup is performed before hydrolysis. In the analysis of peptides in a complex matrix, enzymatic hydrolysis prior to HPLC would result in hydrolysis of all peptides present in a sample, possibly to similar products, making a specific HPLC separation and MS determination of a single specific peptide difficult. Voyksner et al. reported the use of several different immobilized endopeptidases (chymotrypsin, thermolysin, trypsin, V_8 protease) and exopeptidases (carboxypeptidases A, B, and Y), in combination with HPLC/thermospray MS for the identification of synthetic endorphins [142]. Typically, they described immobilized enzyme bioreactors after HPLC separation. Among the endorphins they used, the synthetic α–endorphin contained 16 amino acid residues (M_r 1745), β-endorphin contained 31 residues (M_r 3463), and γ-endorphin contained 17 residues (M_r 1859).

Figure 5.3.4 shows thermospray mass spectra of β-endorphin analyzed after different enzyme bioreactors.

The peptide mapping of recombinant human interferon-gamma (rhIFN-γ) by reversed-phase liquid chromatography with diode-array absorbance and thermospray mass spectrometry has been reported by Legrand et al. [143]. rhIFN-γ (M_r 42,369) extracted from *Escherichia coli* with the addition of an additional N-terminal methionine contains a sequence of 143 amino acids. The digest of rhIFN-γ by *Staphylococcus aureus* strain V_8 endoprotease was directly injected on to a HPLC column with gradient elution (1.2 ml/min) using acetonitrile and 0.1% trifluioroacetic acid/acetonitrile and thermospray MS detection. The total ion current of the eluate of the digest of rhIFN-γ is shown in Figure 5.3.6. The endoprotease hydrolyzes peptide backbones specially at the carboxylic side of glutamic acid and aspartic acid. The amino acid sequence of rhIFN-γ is given in Figure 5.3.5. Both observed cleavage sites for *Staphylococcus aureus* strain V_8 endoprotease and for thermal hydrolysis are also indicated in Figure 5.3.5. The peptide bond at the carboxylic side of aspartic acid is much easier to be hydrolyzed in dilute acid solution especially under the heated conditions during thermospray injection. The mass spectrum in Figure 5.3.7 for peak 21 in Figure 5.3.6 illustrates this mechanism. Besides hydrolysis, spontaneous fragmentation was also observed such as masses 869^{2+}, 927^{2+}, 985^{2+} and 1123^{2+} in Table 5.3.1.

5.4. Applications of CF-FAB

CF-FAB has been the technique most competitive to TSP MS in popularity for the analysis of peptides. The early development of the CF-FAB interface in the analysis of peptides was described by Caprioli [144]. In their studies the sample introduction probe described consisted of a fused-silica capillary (1m x 0.075mm) connected to an injection valve provided with a continuous flow of solvent containing water/glycerol (8:2) and 0.3% of trifluoroacetic acid. Samples might be injected into this flow of solvent or

included in the solvent at a flow rate of about 5 μl/min. 0.3 ng of the peptide substance P (M_r 1347) was analyzed by this system and gave a signal-to-noise ratio of 5:1.

The application of CF-FAB LC-MS for the analysis of proteolytic digests has been described by various groups. Optimization of chromatographic conditions for combined microbore HPLC/CF-FAB MS was described by Caprioli et al.[145]. A 1 x 50 mm column with a postcolumn splitting (1:4) were used to provide chromatographic separations amenable to the flow rates (5 to 10 μl/min) used with the CF-FAB interface. The CF-FAB analysis of proteolytic hydrolysates of human apolipoprotein A-I (M_r 28,100 Da) was described.

The use of large columns with postcolumn splitting offers the advantage of directly coupling of HPLC and mass spectrometry. However, splitting is undesirable when all of the sample is required for FABMS analysis. Henzel et al. reported a packed capillary C18 column (0.32 mm x 150 mm) gradient HPLC-FABMS system [146]. The low flow rates of the packed capillary permitted introduction of the entire column effluent into the mass spectrometer. Detection limits of 0.5-5 pmol were routine. Proteolytic digests of recombinant human methionyl growth hormone and carboxylmethyltransferase were analyzed to evaluate this system. The sequence of met-rhGH is shown in Figure 5.4.1 and the Figure 5.4.2 shows the mass spectrum for one trypsin-digested fragment T1 (MFPTIPLSR) of the protein.

Another application by interfacing packed capillary gradient liquid chromatography to FAB-MS for the analysis of glycopeptide teicoplanin was reported by Coutant et al. [147]. Figure 5.4.3 shows the structures of the teicoplanin A2 group. Figure 5.4.4 and 5.4.5 illustrate the MS total ion current of teicoplanin and the mass spectrum of teicoplanin A2-2 groups respectively.

As discussed previously, the low flow rates of the packed capillary LC chromatography permit introduction of the entire column effluent into the mass spectrometer. Open tubular liquid chromatography (10 μl/min) can also achieve compatibility with FAB MS. In addition to their compatibility of low mobile phase flow

rates, the advantages of packed capillary LC and OTLC over conventional LC (4.6 mm I. d.) are superior chromatographic separation efficiency and the ability to analyze very small samples due to the small injection volumes used. In all of the previous reports, glycerol has been added to the mobile phase at concentrations of 5-15 % to allow compatibility with the FAB ionization process. This imposes severe limitations on the LC in terms of flow-rate, pressure and separation (due to changes in the polarity and viscosity of the mobile phase). The postcolumn addition of the FAB matrix into the LC effluent can prevent the problems of using the FAB matrix as a component of the mobile phase. The method works well in conventional LC, but in capillary LC and OTLC, the mobile phase flow rates are so low, the postcolumn addition of the FAB matrix can cause severe chromatographic peak broadening. Moseley et al. described a method to add FAB matrix using a coaxial sheath with use of open tubular and packed fused-silica capillary LC [148]. By applying this method, independent optimization of both liquid streams can be achieved. In addition, since the FAB matrix and the LC effluent are independently delivered to the FAB probe tip, the band broadening is minimized since the mixing of the matrix and the LC effluent takes place only at the FAB probe tip. Excellent data of this coaxial CF-FAB LC-MS of small peptides were demonstrated. For example, detection of 54 fmole Met-Leu-Phe (M_r 409) is shown in Figure 5.4.6 and the detection of 850 fmole bradykinin (molecular weight 1060) is shown in Figure 5.4.7 using the coaxial CF-FAB MS system.

The performance of the coaxial CF-FAB was further demonstrated with tandem mass spectrometry [149]. As discussed above, this coaxial CF-FAB system is chromatographicly advantageous especially when OTLC columns are employed which offers greater separation efficiency than that of conventional columns, microbore columns and even capillary columns due to the smaller internal diameters of the OTLC columns. In addition, this system is more compatible with mass spectrometry because the smaller internal diameters allow a much lower flow rate. However, there are two obvious disadvantages with CF-FAB mass spectra. First, the CF-FAB mass spectra can be

complicated by the high level of background noise generated from the high concentration of matrix on the probe tip; second, the structurally informative fragment ions are not always observed, especially for small peptides, their fragment ions may be obscured by the matrix ions. By interfacing coaxial CF-FAB with tandem mass spectrometry (MS/MS), the ion of interest is selected in the first MS and fragmented by collisional activation. The fragment ions are subsequently analyzed by the second MS. The matrix ions are excluded and only the parent ions and their daughter ions are detected and the structural information for sequencing is collected. Figure 5.4.8 is the MS/MS spectra for Met-Leu-Phe at 220 pg injection and 22 pg injection. Another MS/MS spectrum of bradykinin is shown in Figure 5.4.9.

5.5. The applicability of ISP and ESP

Electrospray interface and ionization has emerged as a very popular and powerful technique for producing intact ions from large and complex species in solution. ESI also has the capability for very precise molecular weight measurements. This technique makes the power and elegance of mass spectrometric analysis applicable to the large and polar molecules in biological systems [150]. The distinguishing feature of electrospray spectra for large molecules is the sequences of peaks which is the result of the phenomenon of multiple charging, as discussed before, the ions of each peak differing by one charge from those of adjacent neighbors in the sequence (Figure 5.5.1). The multiple charging phenomenon of electrospray ionization has allowed direct molecular weight analysis of proteins at high sensitivity (picomole level) with excellent accuracy and precision. Spectra have been obtained for proteins and peptides having molecular weights up to 133,000, and there is yet no report of an upper limit [151]. In addition, collisional activation of the multiply-charged ions is providing great possibilities for structural analysis of peptides.

As first reported by the group of Fenn [152], the molecular weight of large peptides and proteins may be immediately determined from spectra such as those in

Figure 5.5.1, under several assumptions. First, the adjacent peaks of a series differ by only one charge. Second, the charging is due to proton attachment to the molecular ion. These assumptions have been proved excellent for nearly all proteins studied to date where alkali attachment contributions are small. Under those assumptions, Eq. 5.5.1 describes the relationship between a multiply charged ion at m/z-value p_1 with charge z_1 and molecular weight M_r

$$p_1 z_1 = M_r + 1.0079 z_1 \qquad \text{(Eq. 5.5.1)}$$

The molecular weight of a second multiply protonated ion at m/z-value p_2 (where $p_2 > p_1$) that is r peaks away from p_1 (e.g., r = 1 for two adjacent peaks) is given by

$$p_2(z_1 - r) = M_r + 1.0079(z_1 - r) \text{(Eq. 5.5.2)}$$

Eq. 5.5.1 and Eq. 5.5.2 can be solved for the charge of p_1

$$z_1 = r(p_2 - 1.0079) / (p_2 - p_1) \qquad \text{(Eq. 5.5.3)}$$

The molecular weight is calculated by using the nearest integer value of z_1 from Eq. 5.5.3 in Eq. 5.5.1.

A wide variety of peptides and proteins have been characterized by ESP in hundreds of publications. There are usually three significant features in a typical electrospray mass spectrum of a tryptic peptide as shown in Figure 5.5.1. First, higher protonated molecular ions have a lower value of m/z and lower protonated molecular ions have a higher value of m/z. In most cases, a singly-charged protonated ion has very low intensity. Peptides are presumably protonated on the amino terminus and on the basic amino acid residues such as lysine, arginine and histidine. Second, there is very low background chemical noise. The low abundance of background ions is due to the high drying or desolvation efficiency of the countercurrent flow of warm nitrogen. Third, the sensitivity is impressive. Electrospray can provide excellent sensitivity at low pmole level.

Collisional activation of the multiply charged ions, either in the region between the nozzle and skimmer or in the collision cell of a tandem mass spectrometer provides the possibility to "fingerprint" proteins from various sources [153,154]. Experiments have been performed by increasing the potential difference between the nozzle and the skimmer in the electrospray interface [155,156]. The results of those experiments have demonstrated that fragmentation is induced at high potential. The efficiency of ion-molecule collisions can be enhanced by applying a small potential difference between two ion sampling plates, i.e., between the nozzle and the skimmer. By increasing the potential difference between the nozzle and the skimmer the excess internal energy that is gained by the molecules can be increased and fragmentation is induced. The combination of electrospray with triple-stage quadrupole MS/MS appears to be another promising technique for peptide sequence determination at the low picomole level [157]. The mass spectra of collision-induced dissociation (CID) in the collision cell of a tandem mass spectrometer can readily be correlated with the peptide sequence for peptides with molecular weights up to 5,000. For larger peptides sequencing is more difficult due to ambiguities resulting from the limited resolution as well as the absence of product charge state information [156].

The applicability of ISP in the analysis of peptides is similar to that of ESP since similar spectra can be obtained for peptides and proteins with both techniques. Ionspray is characterized as a nebulizer-assisted ESP.

The electrospray (including ionspray) interface and ionization is now widely used in characterization of peptides and proteins and is almost routine in this field only about six years after the initial reports of Fenn et al. In the following section, several applications will be presented.

Tryptic digests of native recombinant human growth hormone (rhGH) and reduced carboxymethlated tissue plasminogen activator (rt-PA, a recombinant glycoprotein) were studied by Mock et al. using a precision-flow microbore HPLC system coupled to an electrospray single-stage quadrupole mass spectrometer [158]. Separations

were carried out on a Reliasil column (15cm x 1mm) packed with a C18 stationary phase with gradient elution of aqueous 0.1% trifluoroacetic acid and acetonitrile/0.085% TFA (Figure 5.5.2). Collisionally activated dissociation spectra of peptides were generated between the capillary and the skimmer (Figure 5.5.3). The voltages were increased from 36 to 100V on the capillary lens and to 195 from 136 for the tube lens. The PEPMATCH and PEPMAP programs were used to interpret the data by identification of predicted peptides, incompletely digested peptides and unusual peptide cleavages. Peptide mapping at the low pmol level was achieved.

Schindler et al. have described their results for the analysis of hydrophobic proteins and peptides by electrospray-ionizaition mass spectrometry [159]. Hydrophobic proteins (subunits VIIIa and VIIIb of cytochrome-c oxidase, subunit A6L of ATP synthase, the 6.5kDa subunit of the bc_1 complex, a geranylgeranyl-modified peptide, bacteriorhodopsin and cytochrome b_5) were dissolved in an electrospray carrier solvent of either $CHCl_3$/methanol/H_2O(2:5:2, v/v/v) (CMW 2:5:2) containing 1% acetic acid or $CHCl_3$/methanol/0.1% aqueous trifluoroacetic acid (4:4:1, v/v/v) (CMW 4:4:1). After centrifugation, portions (40-300 pmol of protein) were analyzed by electrospray-ionization MS on a VG Bio-Q instrument with an atmospheric-pressure electrospray ion source and a quadrupole mass analyzer with a maximum mass range of 4000. Mass spectra for both cytochrome b_5 and bacterioopsin are shown in Figure 5.5.4 and Figure 5.5.5.

Determination of the primary structure of porcine neutrophil peptides (PNP) by electrospray LC/MS has been reported by Mirgorodskaya et al. [160]. For peptide mapping, the individual PNP were separated by HPLC. After reduction and alkylation with 4-vinylpyridine, each PNP was digested with pepsin and the products were analyzed by the ESP LC/MS. For sequencing, the digestion products were hydrolyzed with heptafluorobutyric acid before analysis. Definite structural fragments were identified and their locations determined.

Mixtures (1-2 mg) of 48 synthetic octapeptides in equimolar amounts, each with five defined and three variable residues, were dissolved in 1 ml of acetonitrile/aqueous1% formic acid (1:1). The analysis of the solution by positive-ion electrospray MS on a Sciex API III triple-quadrupole instrument equipped with a nebulizer assisted electrospray source (spray needle held at +4.8 kV) and by tandem-MS has been studied by Metzger et al. [161]. Collision-induced dissociation experiments were performed using Ar with collision energies of 30-60 eV. For the structurally similar peptides mass discrimination effects were found. The ion intensity distribution of the protonated molecular ions was independent of concentration. The peak heights in the electrospray spectra reflected the number of isobaric peptides in the mixture. Tandem-MS identified by-products which were formed by incomplete removal of side chain protecting groups during peptide synthesis. Daughter ion scans identified common structural features and parent ion and constant neutral loss scans detected peptides modified with the same protecting group.

Reversed-phase capillary HPLC with an on-line detection system in the analysis of peptides and proteins has been reported by Giordani et al. [162]. Peptide mixtures were first separated by reversed-phase HPLC on a C_{18} column (15 cm x 0.5 mm i.d.) equipped with a stainless-steel pre-column splitter with gradient elution using aqueous acetonitrile. The components were then analyzed using an array of three detection systems in series: UV detection (214 nm), fluorescence detection at 356 nm (excitation at 280 nm) and electrospray ionization mass spectrometry (ES-MS). The on-line combinations of detectors following chromatographic separation by HPLC provides complementary information which was useful in the characterization of proteins/peptides. The fluorescence detection of tryptophan containing peptides provided selectivity in peptide mapping while UV absorption data at 214 nm enabled detection of the peptide bond and ES-MS data provided unambiguous measurements of molecular masses. The application of this system was demonstrated in the analysis of proteolytic digests of bovine serum albumin (BSA, MW 66,500) and stromelysin catalytic domain (SCD, MW 19,492) protein expressed in *E. coli*. Fig. 5.5.6 shows the UV and fluorescent

chromatographic profiles of analysis of the tryptic digest of expressed SCD, and Fig. 5.5.7 shows the electrospray mass spectra for certain residues

5.6. Conclusion

From the results reviewed in the above sections, it can be concluded that significant progress has been achieved in this field. Among LC-MS techniques, ESP, TSP and CF-FAB are the most popular interfaces in dealing with peptides and proteins today.

Both MBI and DLI have been applied in the analysis of derivatized and underivatized amino acids and small peptides with some success as discussed above, but these interfaces suffer from some practical problems i.e., sensitivity, thermal degradation and "tuning" variability. In addition, both MBI and DLI are unable to deal adequately with less volatile samples such as large peptides and proteins. Today both DLI and MBI are almost abandoned.

The CF-FAB is least susceptible to those above mentioned problems. CF-FAB has become a frequently used method, since it allows for the analysis of high molecular weight compounds by LC/MS and additionally offers some advantages over conventional FAB techniques. CF-FAB is most compatible with the use of lower flow packed microbore columns. However, CF-FAB has limited application for less polar compounds.

TSP has demonstrated its capability in analysis of peptides. TSP is ideally suited to reversed-phase LC with conventional packed columns that is currently the most widely used separation technique. The disadvantages of thermospray are the variation in sensitivity from compound to compound and the lack of fragment ion information in many cases. In addition, thermal decomposition for some compounds has also been observed. At present both CF-FAB and TSP seem to be declining in popularity in the analysis of peptides and proteins.

Significant improvements in LC-MS interfaces have been achieved from various atmospheric-pressure ionization interfaces, i.e., ESP, ISP. Both ESP and ISP are reliable techniques for very high molecular weight samples. One of the general problems that has to be faced in biochemical applications is limited sample availability. For LC-MS applications this may have importance consequences. ESP as well as TSP are capable of generating mass spectra of proteins like myoglobin. But, TSP apparently is considerably less sensitive than ESP. For example, 100-400 pmoles of protein are needed in TSP, while 1-50 pmoles are generally sufficient in ESP. Although the sample concentration needed for the mass spectrum may be similar with both techniques, in practice the analysis is often limited by the available amount of sample, because in many biochemical preparations it is difficult to produce or isolate large amounts of sample. It can also be concluded that the overall ionization efficiency in TSP is less than in ESP. More analyte molecules are needed in TSP than in ESP to achieve a reasonable signal-to-noise ratio in the spectrum. As a result, ESP is the technique of choice over TSP in most biochemical applications.

Electrospray ionization mass spectrometry offers several significant advantages over CF-FAB-MS as a detection method for liquid chromatography. For ES-MS, there is no need to require modification of the mobile phase prior to mass spectrometric detection. However, CF-FAB-MS requires a viscous matrix, usually glycerol, at 1-5% in the mobile phase. If a viscous matrix is added either prior or post to the column, additional band broadening occurs in the chromatographic separation. Although not as simple to manufacture, a coaxial design overcomes this problem. However, there still may be memory effects which degrade the total ion current (TIC) profile obtained from the mass spectrometer.

Electrospray ionization mass spectrometry also offers advantages over CF-FAB-MS in the analysis of large peptides and glycopeptides. Detection limits with CF-FAB-MS decrease for peptides with M_r greater than 3000. For example, Hemling et al. [163] reported that following HPLC separation, glycopeptides with an M_r of 3000-4000 at the

50 pmol level were readily analyzed by ES-MS but CF-FAB failed to detect them. However, CF-FAB-MS with a coaxial interface design apparently overcomes this limitation in the analysis of high mass peptides [164].

Because CF-FAB generates singly and perhaps doubly protonated molecules, the limiting factor in CF-FAB-MS analysis of large peptides can be the mass-to-charge (m/z) range of the instrument. In contrast to CF-FAB, electrospray ionization generates multiply protonated molecules which enables conventional mass spectrometers (quadrupoles, sectors) with limited m/z ranges (*e.g.* 4000 or less) to measure molecular masses of molecules with M_r >100,000. The ability to analyze peptides and proteins at the low pmol level, along with the ease with which electrospray ionization mass spectrometry can be interfaced to capillary and microbore HPLC has made it a highly suitable interfacing technique for LC/MS in the analysis of peptides in the last five years, and today it has become the major technique used in the peptide and protein field.

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Table 2.2.1. Proton affinities of some compounds

base	proton affinity (in kj/mol)
H_2	422
CH_4	536
H_2O	723
i-C_4H_8	823
NH_3	857
CH_3NH_2	894
pyridine	921

Table 5.3.1. The results of the interpretation for the mass spectrum in Fig. 5.3.7.

Retention time (min)	Masses found $(M + xH)^{x+}$	Fragments identified	(M + H) theoretical
41.75 (peak 21)	410^{+}	91-93	409.4
	$869^{2+}/877^{2+}$	77-90	1756.1
	$927^{2+}/936^{2+}$	76-90	1871.1
	985^{2+}	76-91	1986.2
	1074^{2+}	77-93	2147.4
	754^{3+}, $1123^{2+}/1132^{2+}$	76-93	2262.5
	975		
	998		

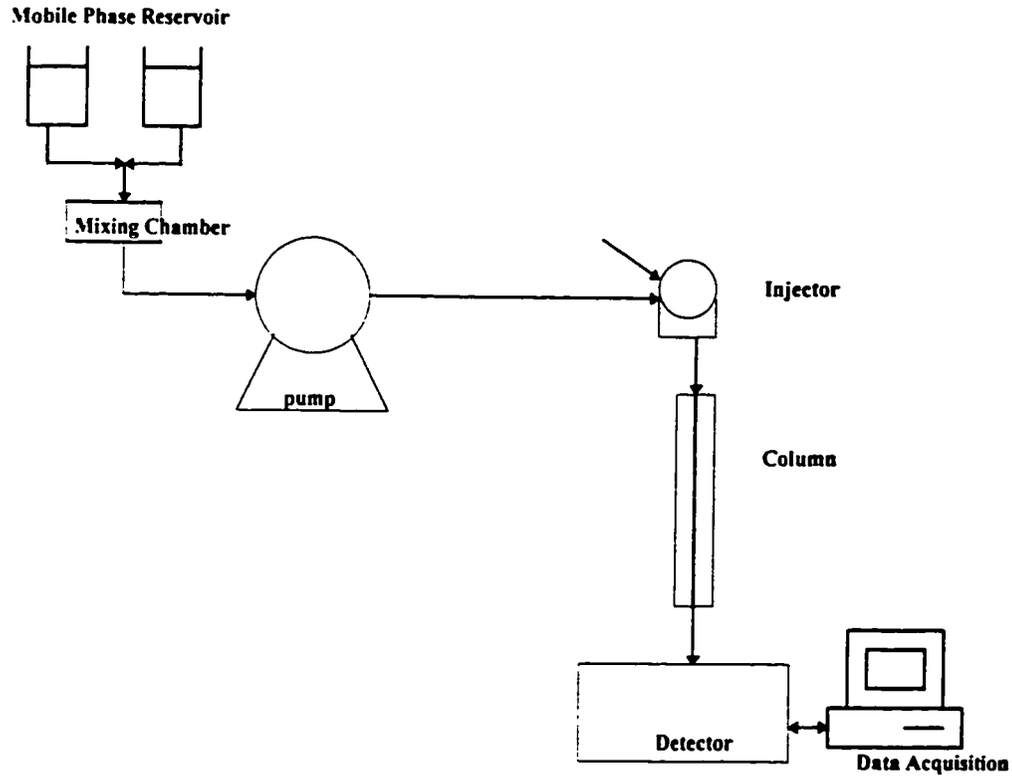
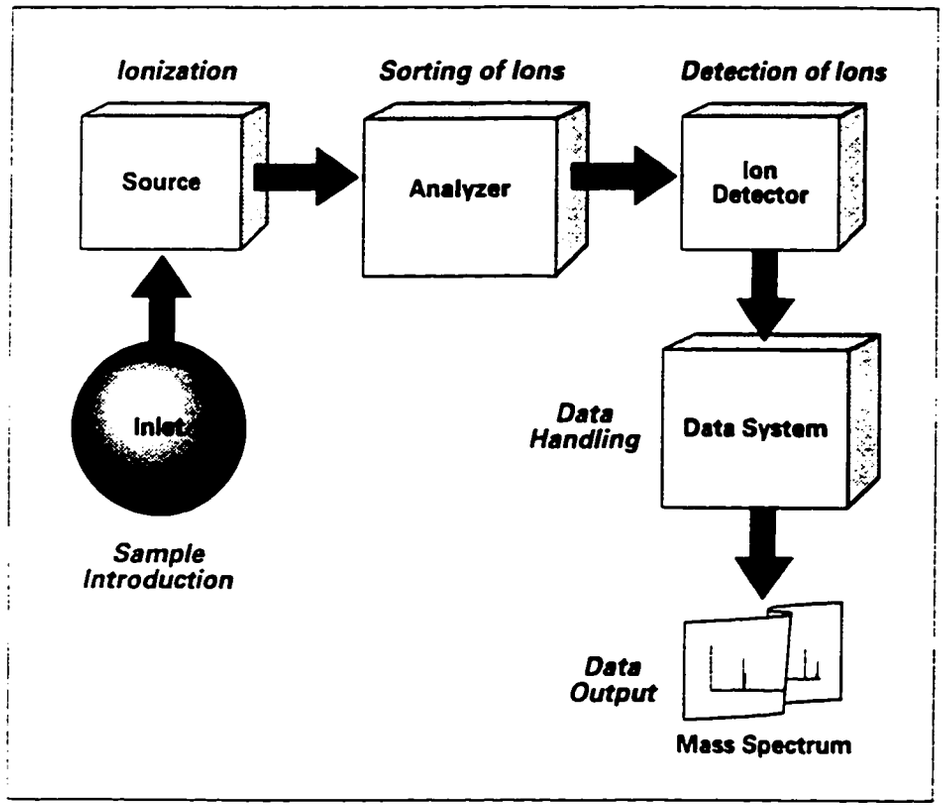


Figure 1.1.1. Basic configuration of a high-performance liquid chromatography.



The components of a mass spectrometer.

Figure 2.1.1. The components of a mass spectrometer.

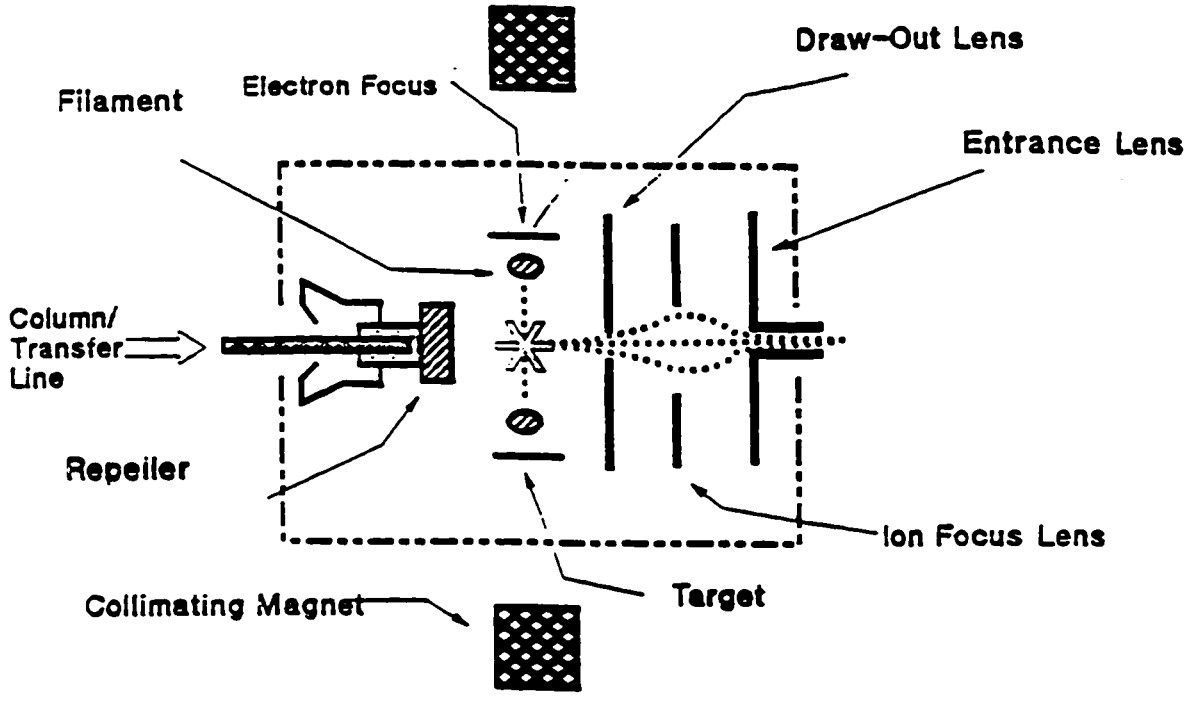


Figure 2.2.1. Electron-impact ion source and ion-accelerating system.

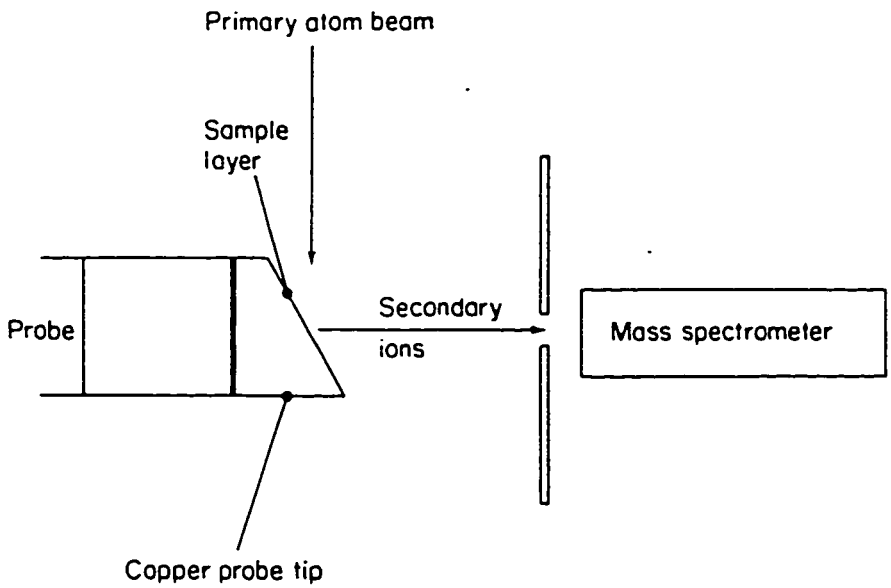


Figure 2.2.2. Schematic representation of a FAB source.

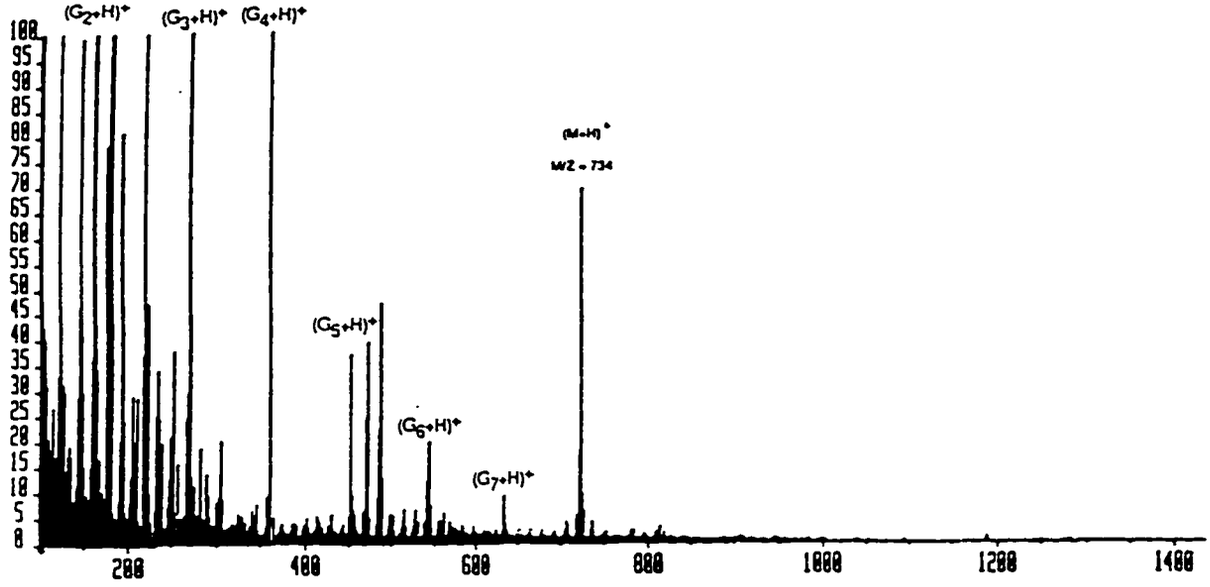


Figure 2.2.3. Mass spectrum of 6 pmol of dipalmitoyl phosphatidyl choline obtained using a fast atom bombardment source. (Reprinted with permission from ref. 28. Copyright (1989) American Chemical Society.)

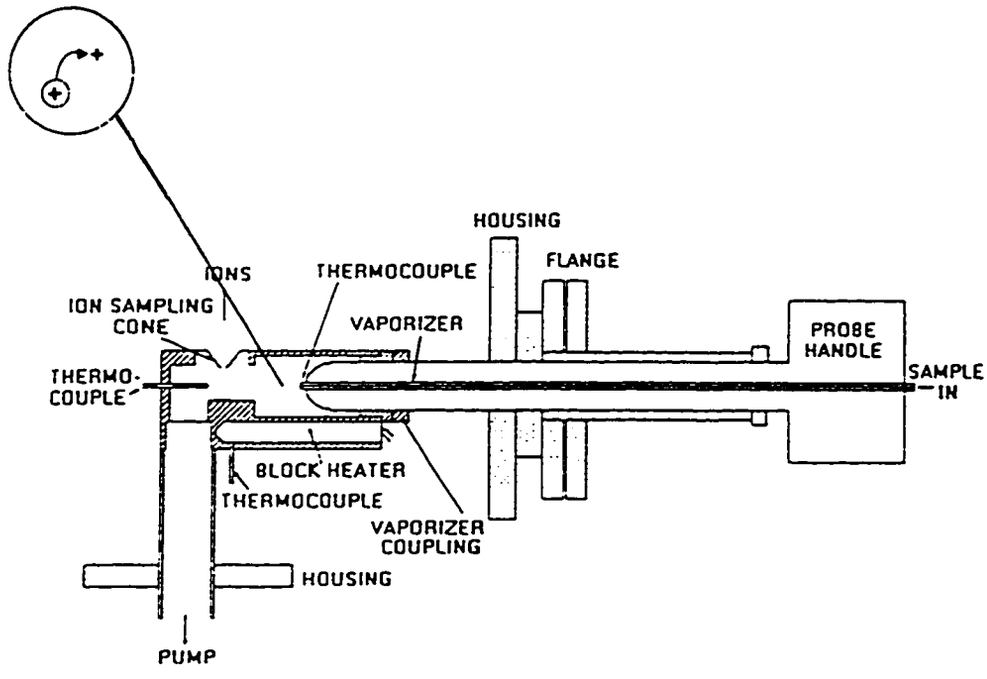


Figure 2.2.4. Schematic representation of a TSP ionization source. (Reprinted with permission from ref. 91. Copyright (1989) Elsevier Science.)

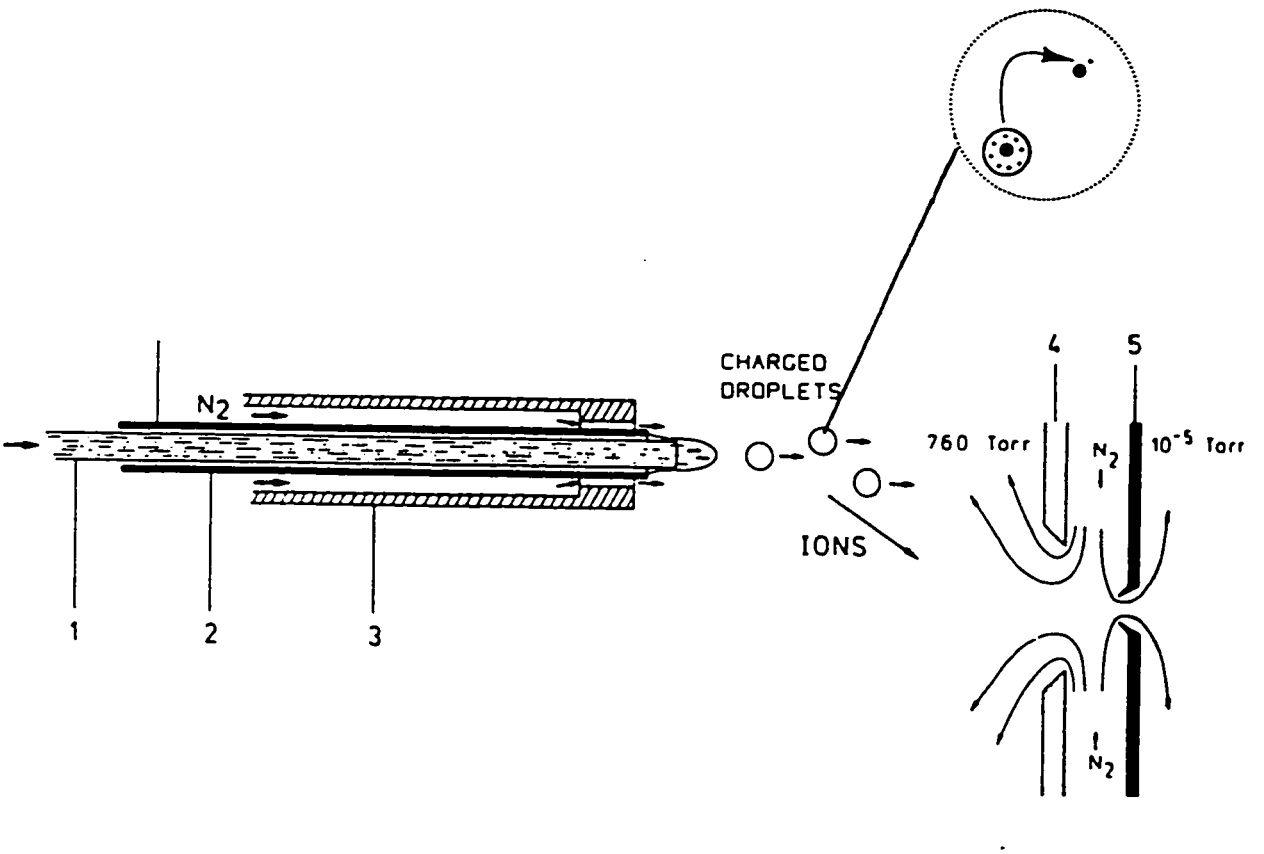


Figure 2.2.5. Schematic representation of ESP and ISP ionization source. (Reprinted with permission from ref. 128. Copyright (1987) American Chemical Society.)

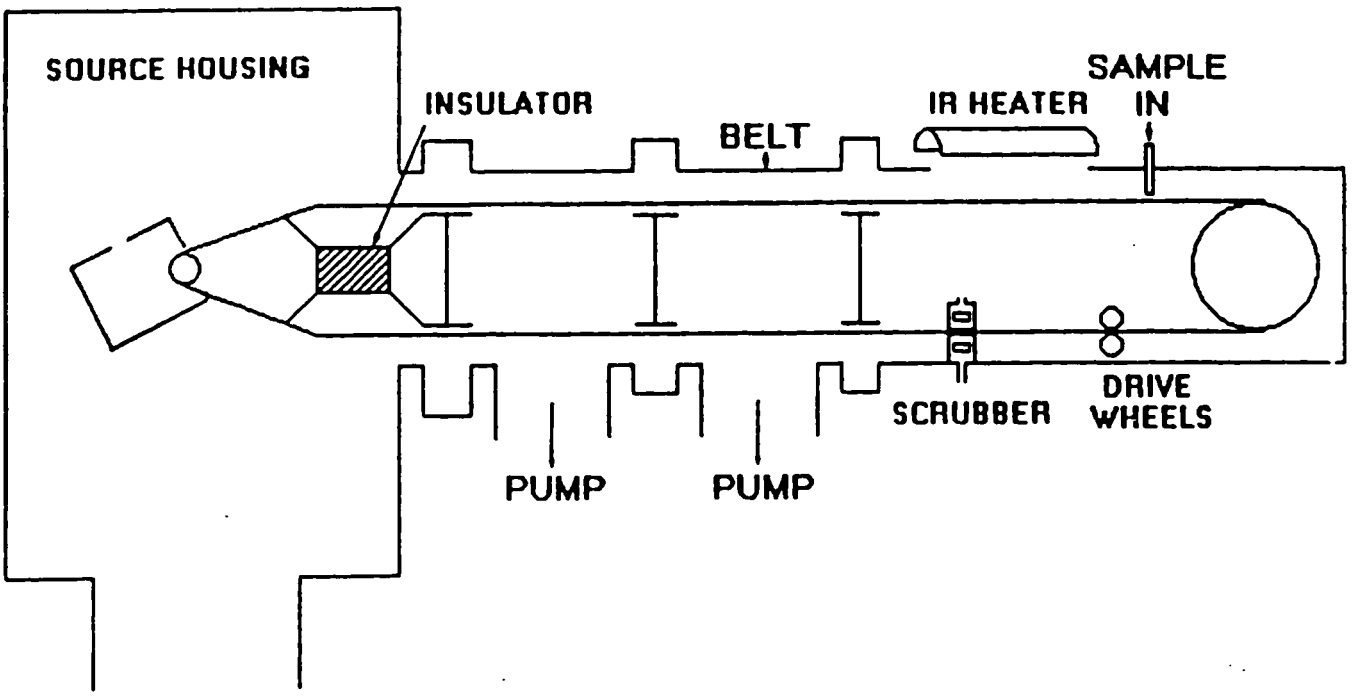


Figure 4.1.1. A moving-belt interface. (Reprinted with permission from ref. 60. Copyright (1976) Elsevier Science.)

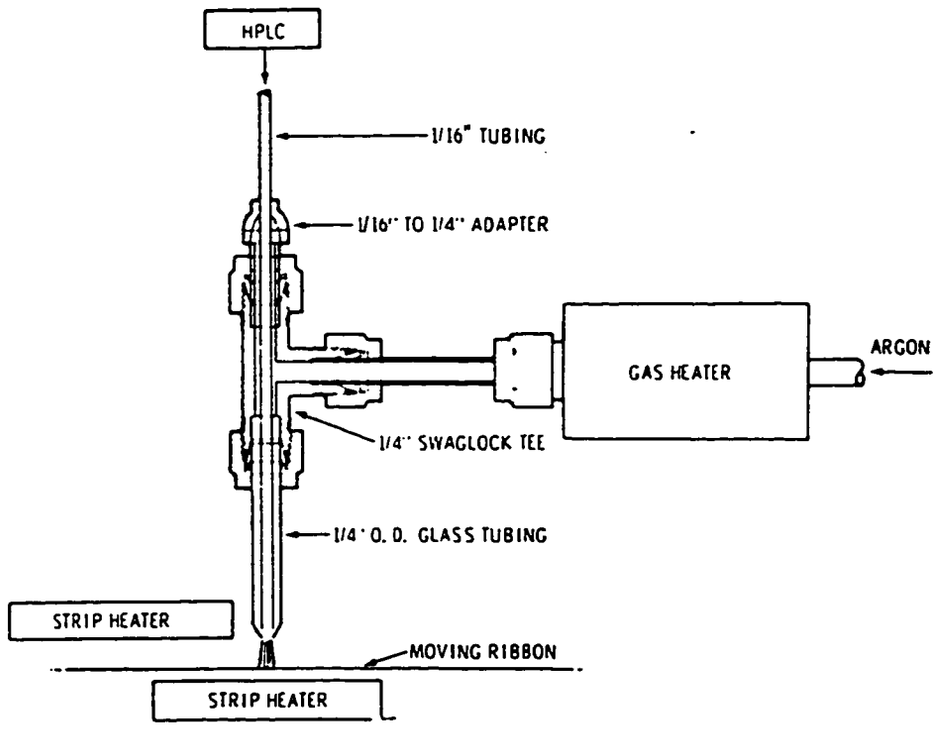


Figure 4.1.2. A heated gas pneumatic nebulizer. (Reprinted with permission from ref. 63. Copyright (1981) American Chemical Society.)

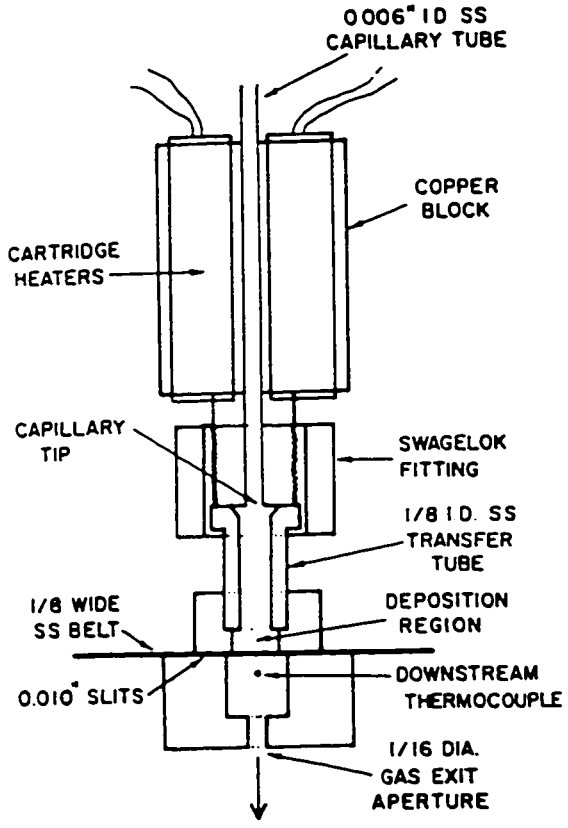


Figure 4.1.3. A thermospray solvent deposition nebulizer. (Reprinted with permission from ref. 67. Copyright (1984) American Chemical Society.)

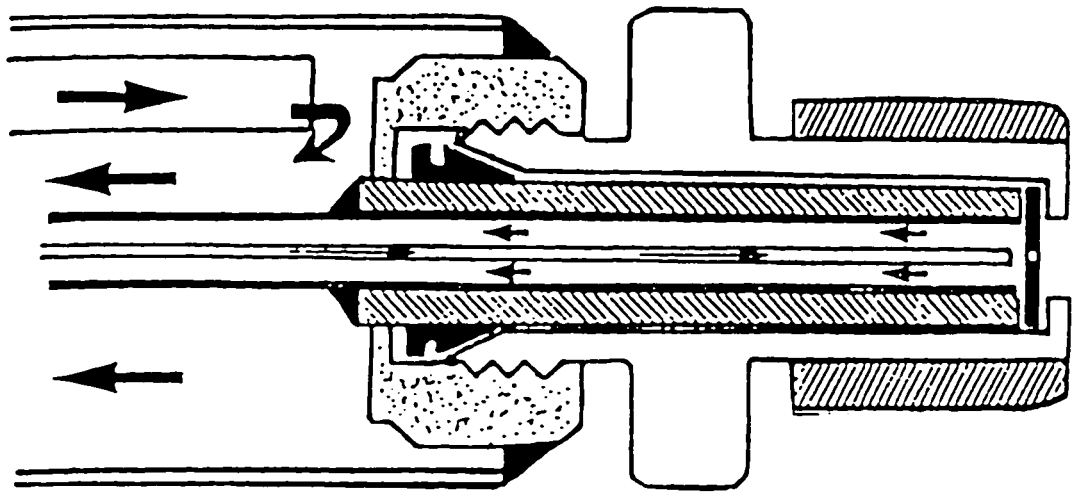


Figure 4.2.1. A DLI interface probe with a diaphragm. (Reprinted with permission from ref. 75. Copyright (1981) Elsevier Science.)

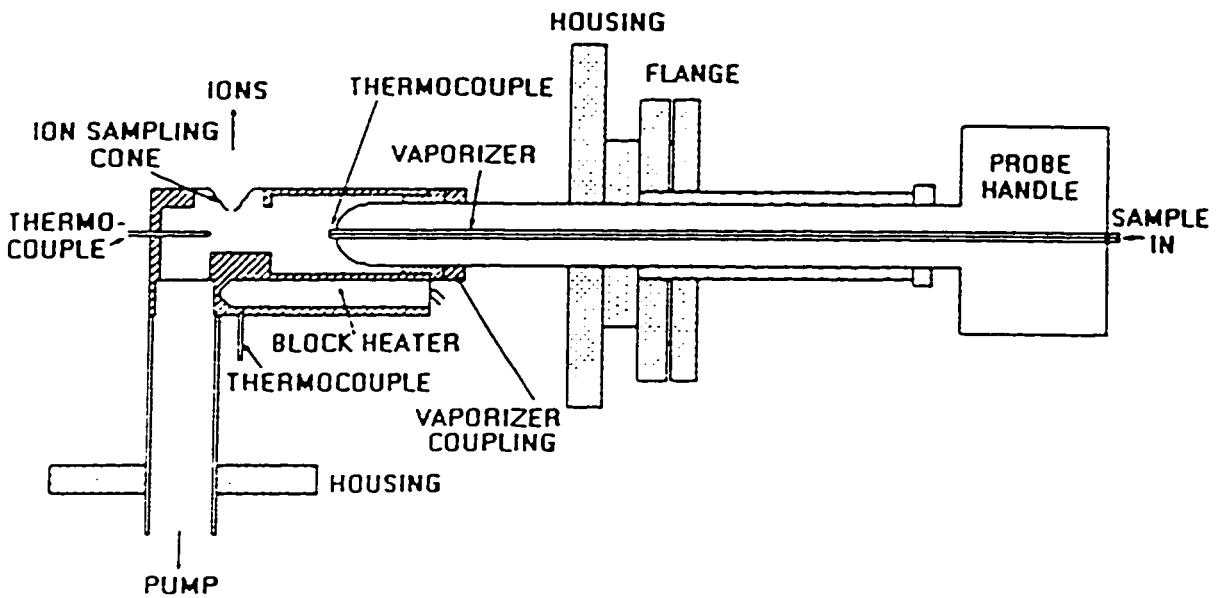


Figure 4.3.1. A thermospray interface. (Reprinted with permission from ref. 91. Copyright (1989) Elsevier Science.)

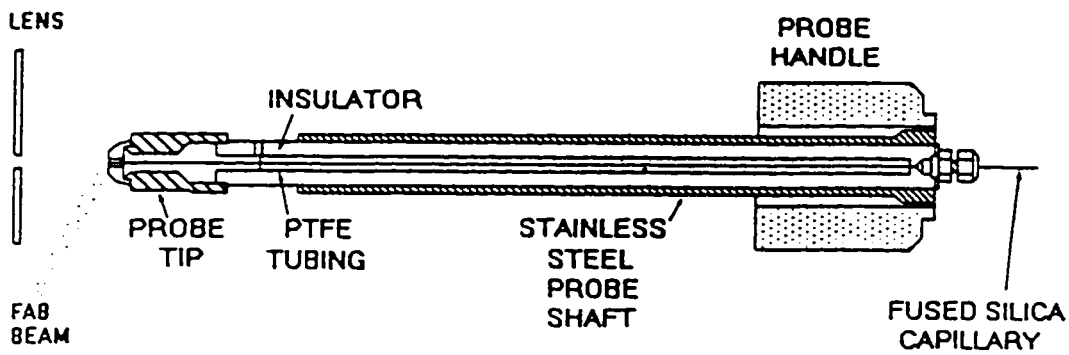


Figure 4.4.1. A CF-FAB interface. (Reprinted with permission from ref. 108. Copyright (1989) Elsevier Science.)

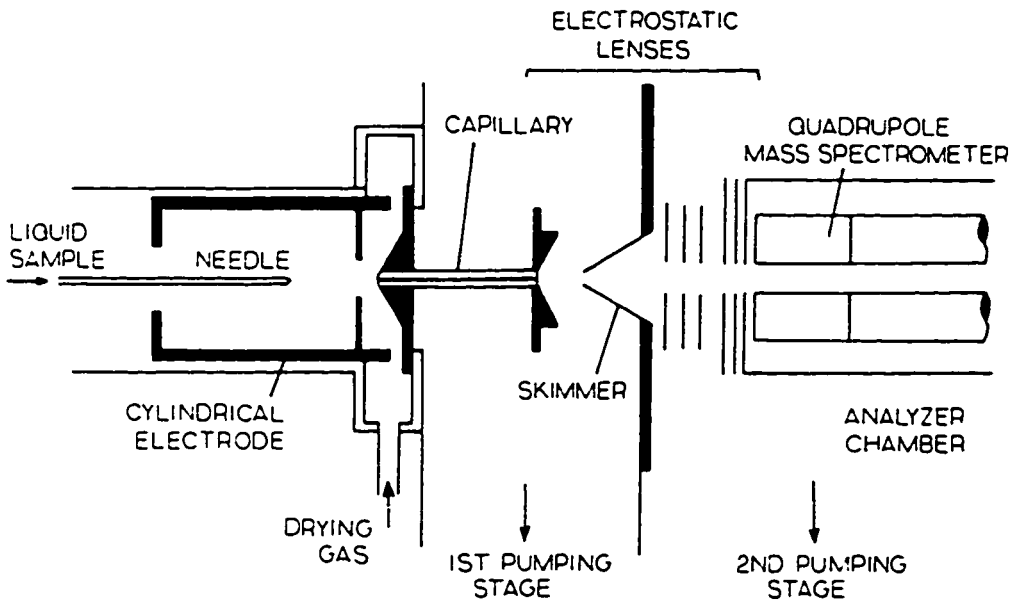


Figure 4.5.1. An electrospray interface for LC-MS. (Reprinted with permission from ref. 117. Copyright (1985) American Chemical Society.)

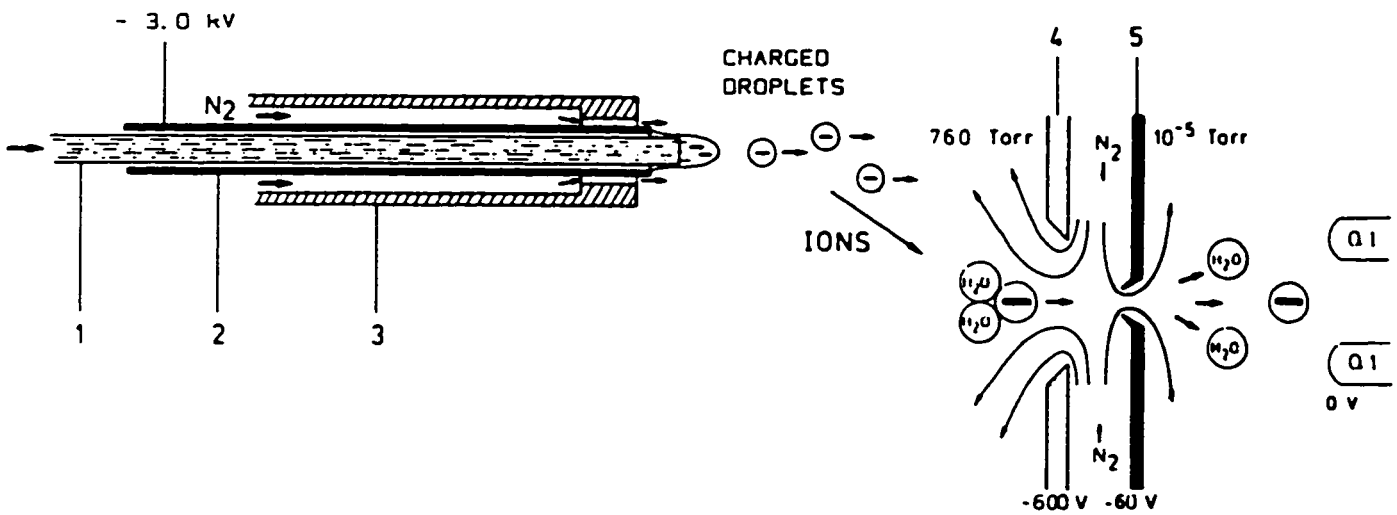


Figure 4.5.2. An ionspray interface. (1) 50 μm ID fused-silica capillary, (2) 200 μm ID stainless steel capillary, (3) 0.8 mm ID PFTE tubing with PFTE insert at the top, (4) ion focusing lens and counter electrode in ISP, (5) plate with 100 μm ID conical sampling orifice. (Reprinted with permission from ref. 128. Copyright (1987) American Chemical Society.)

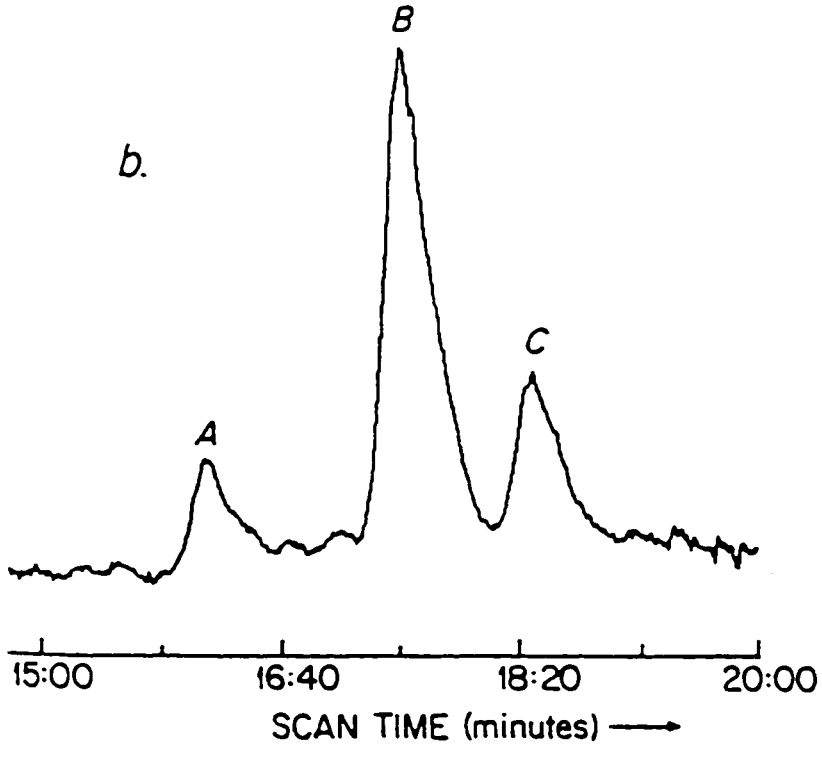


Figure 5.1.2. HPLC-MS chromatograms of permethylated Met-enk. Peaks: A=Ac-Me-Tyr-Me-Gly-Me-Gly-Me-Phe-OMe; B=Met-enk [Ac-Me-Tyr-Me-Gly-Me-Gly-Me-Phe-Me-Met-OMe]; C=C-methylate Met-enk. (Reprinted with permission from ref. 132. Copyright (1984) Elsevier Science.)

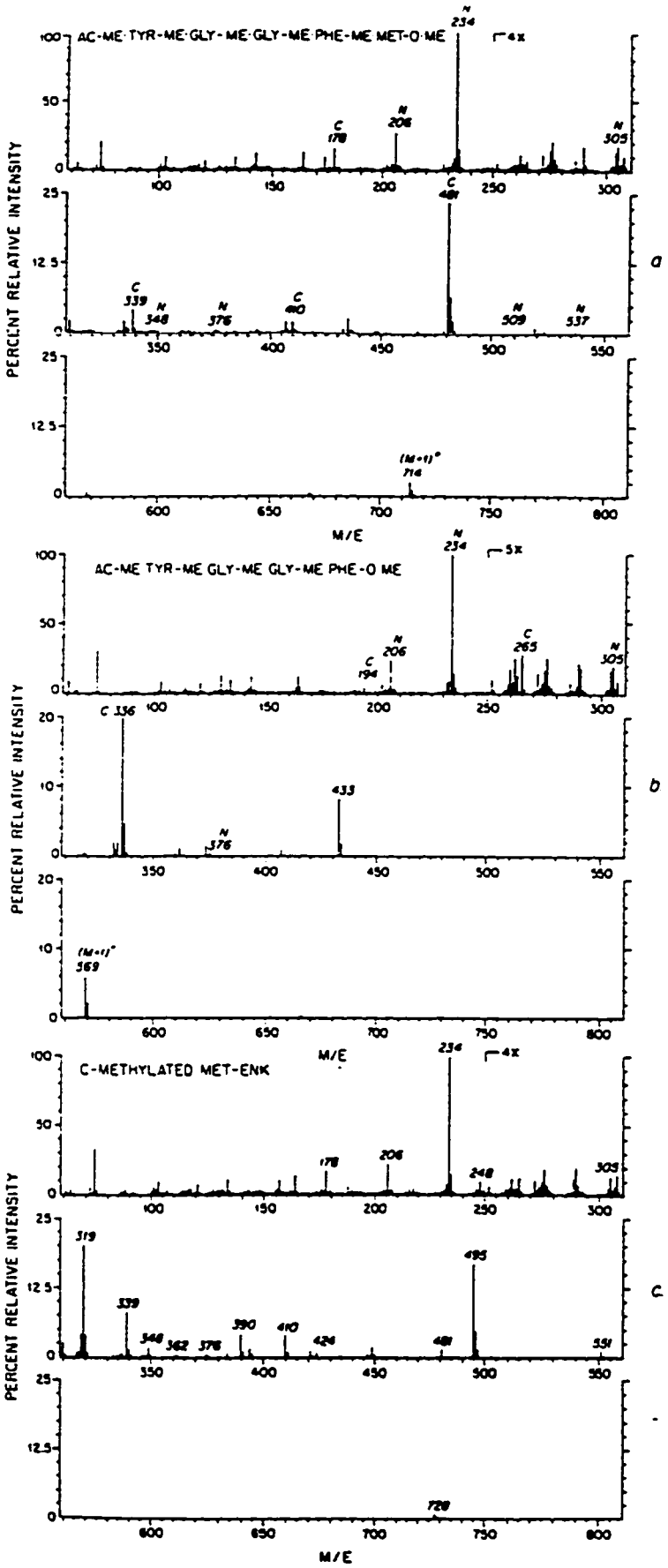


Figure 5.1.3. Mass spectra (CI-isobutane) of (a) permethylated Met-enk (b) Ac-Me-Tyr-Me-Gly-Me-Phe-OMe and (c) C-methylated Met-enk from HPLC-MS chromatogram of Figure 5.5.2. (Reprinted with permission from ref. 132. Copyright (1984) Elsevier Science.)

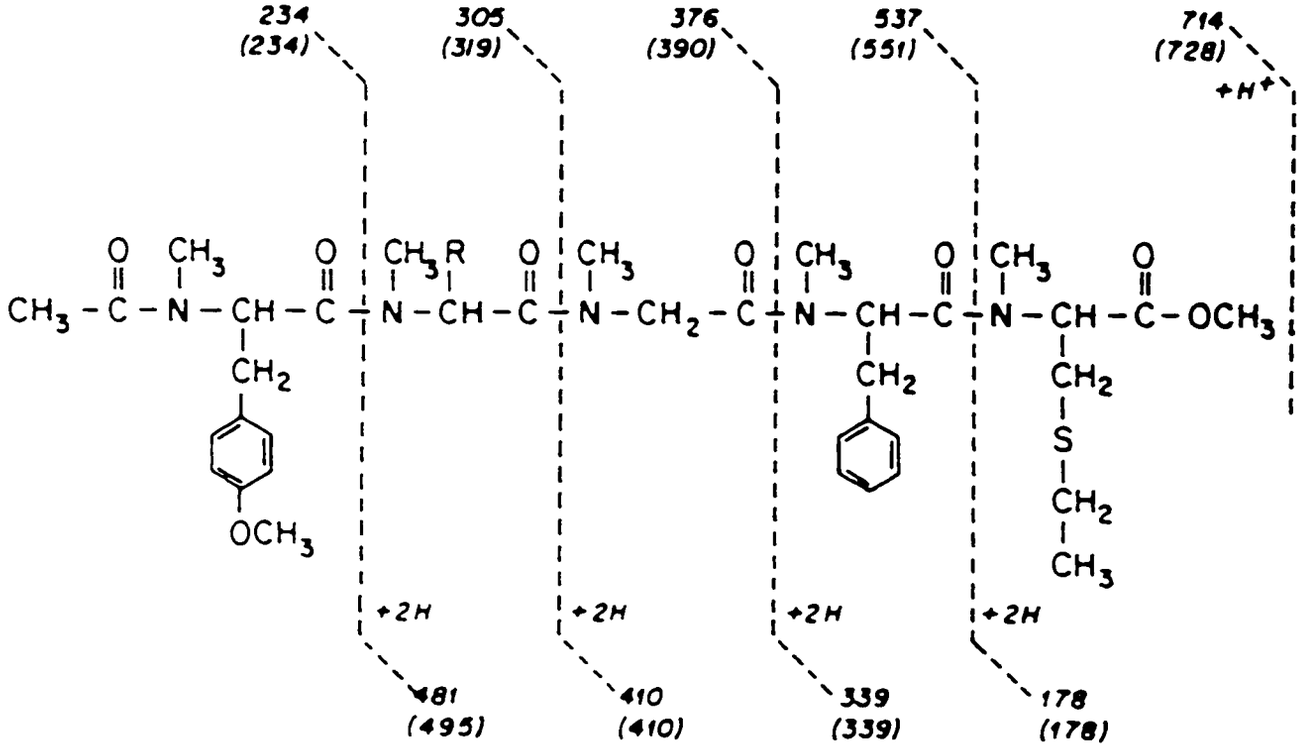


Figure 5.1.4. Fragmentation (CI-isobutane) of N-acetyl-permethylated Met-enk and C-methylated derivative of the Gly residue attached N-terminal Tyr. (Reprinted with permission from ref. 132. Copyright (1984) Elsevier Science.)

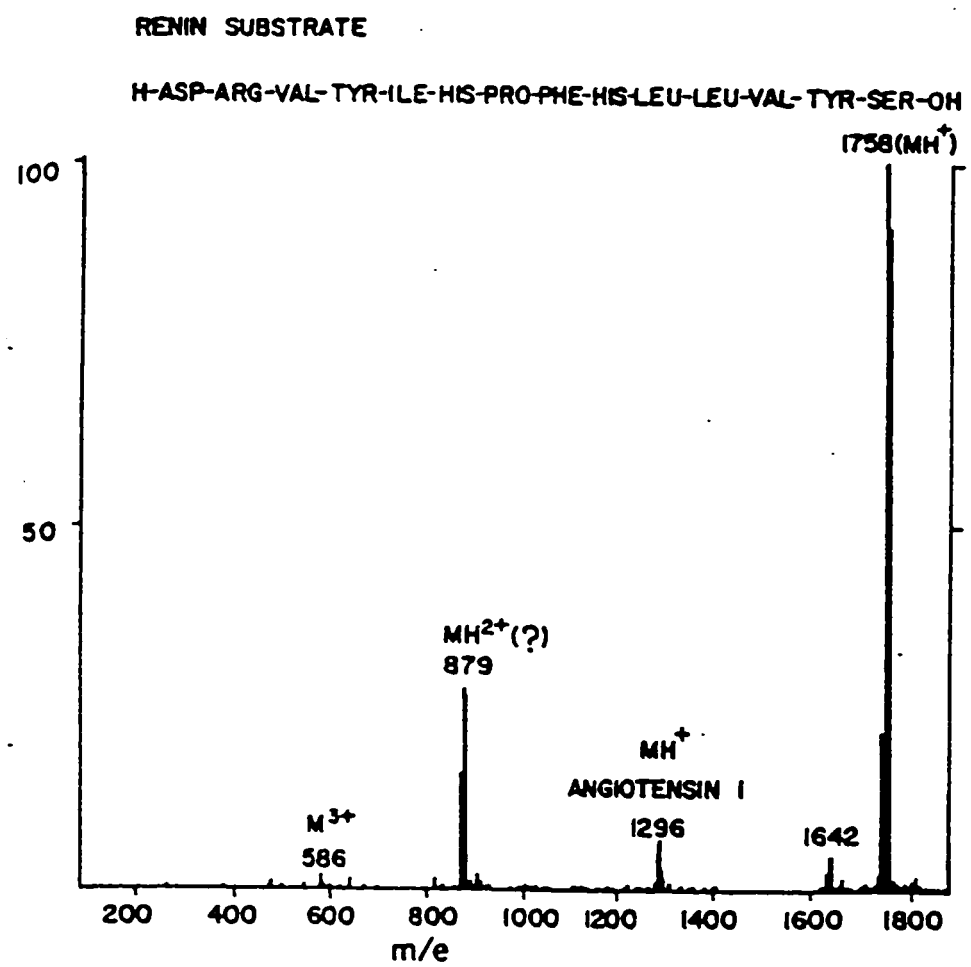


Figure 5.3.1. Thermospray ionization mass spectrum of renin substrate, M_r 1757. (Reprinted with permission from ref. 138. Copyright (1983) American Chemical Society.)

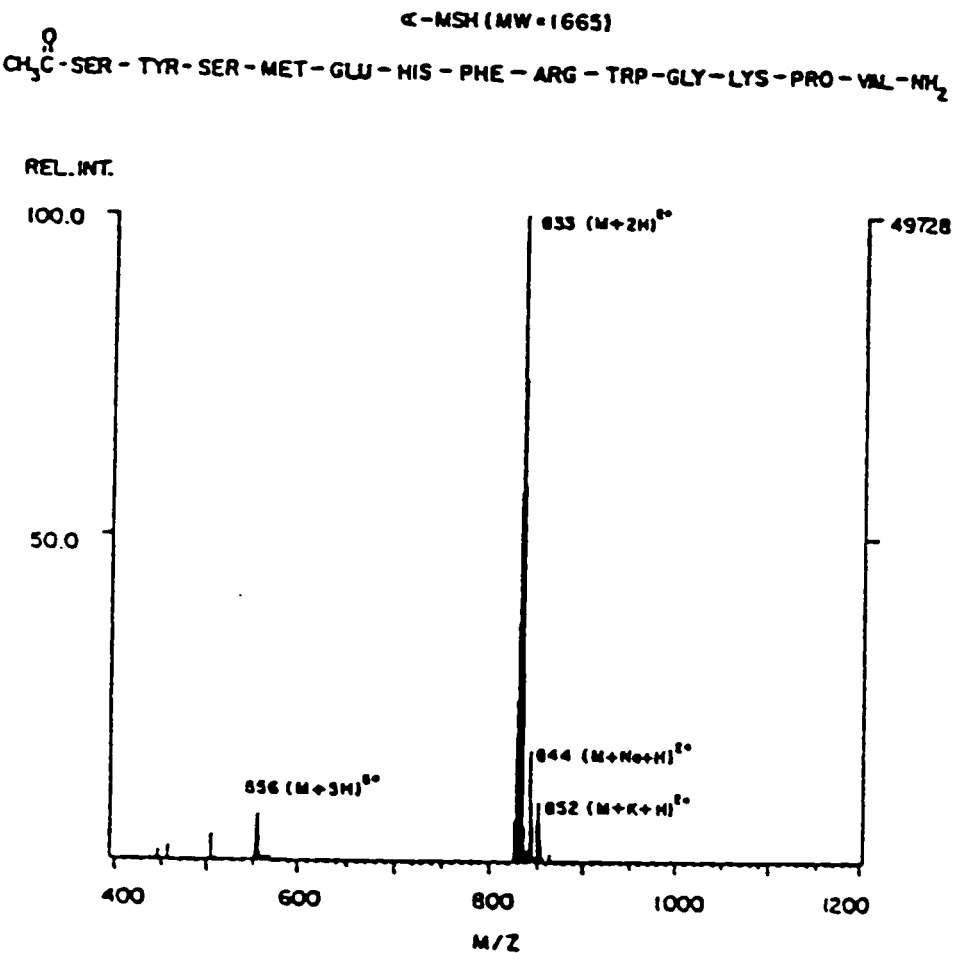


Figure 5.3.2. Mass spectrum of α-MSH without tryptic hydrolysis. (Reprinted with permission from ref. 139. Copyright (1984) American Chemical Society.)

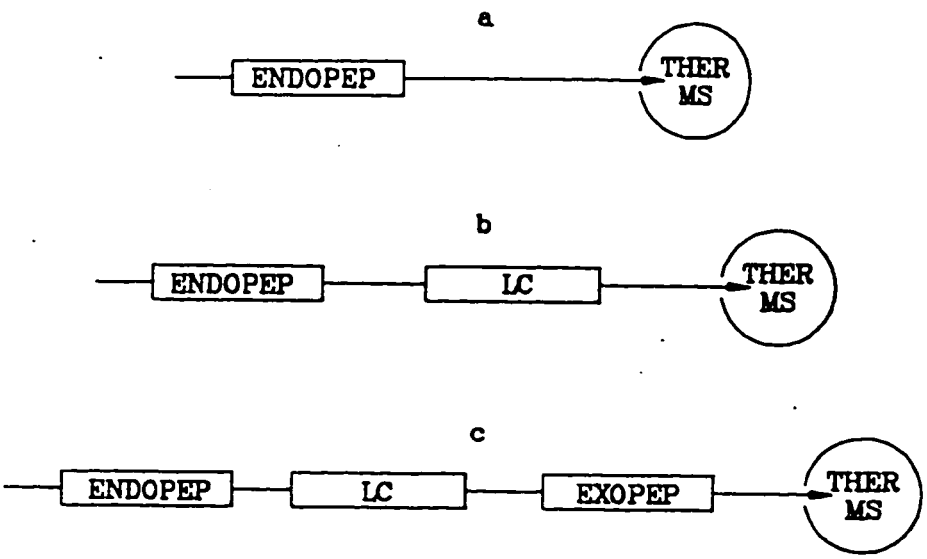


Figure 5.3.3. Different configurations of the enzyme-thermospray LC-MS sequencing instrumentation. (Reprinted with permission from ref. 140. Copyright (1988) American Chemical Society.)

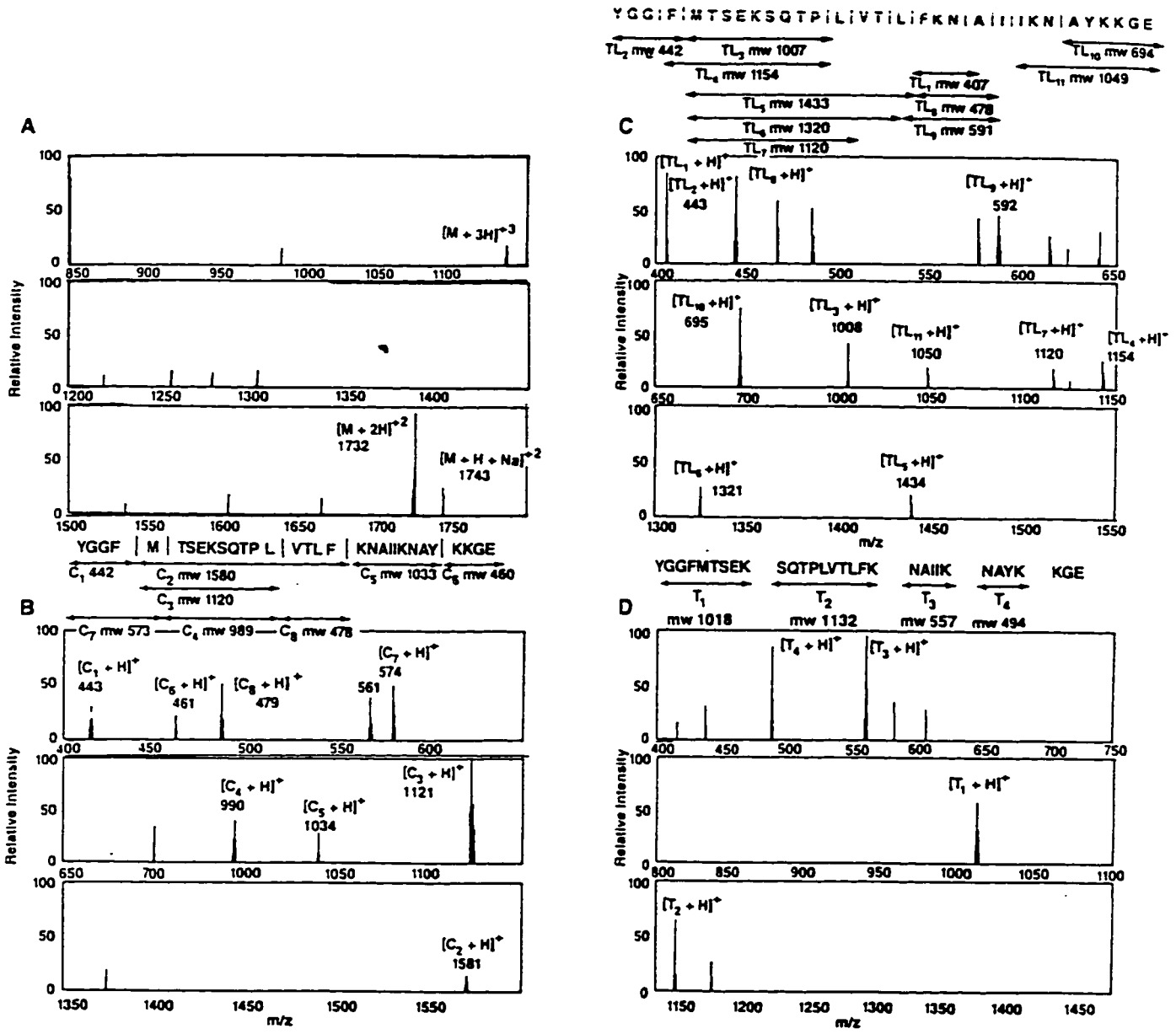


Figure 5.3.4. Thermospray mass spectra of β-endorphin (A) analyzed directly without hydrolysis, (B) after one pass through a chymotrypsin bioreactor, (C) after one pass through a thermolysin bioreactor, (D) after one pass through a trypsin bioreactor, and (E) after one pass through a V_8 protease bioreactor. All spectra were acquired for 1nmol injection of β-endorphin in aqueous 0.1 M ammonium acetate under identical conditions. (Reprinted with permission from ref. 142. Copyright (1990) Academic Press.)

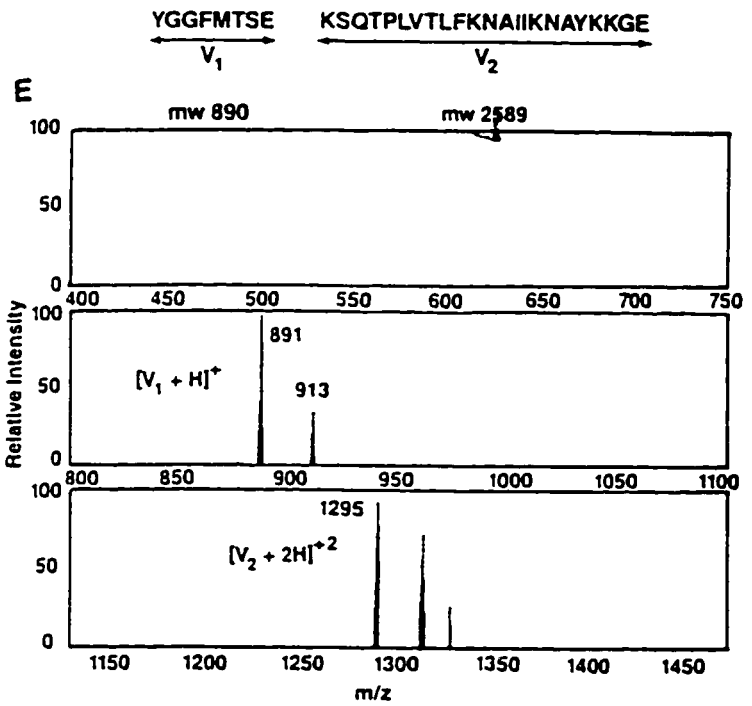


Figure 5.3.4. (Continued) Thermospray mass spectra of β-endorphin (E) after one pass through a V_8 protease bioreactor.

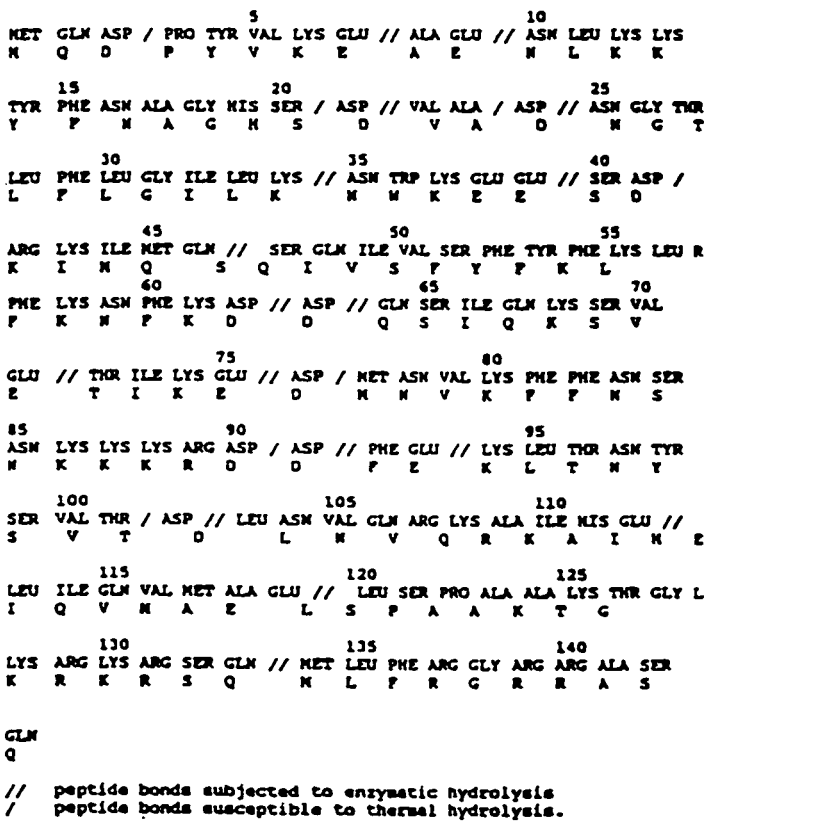


Figure 5.3.5. Observed enzymatic and thermal cleavage sites of rhIFN-γ. (Reprinted with permission from ref. 143. Copyright (1993) Elsevier Science)

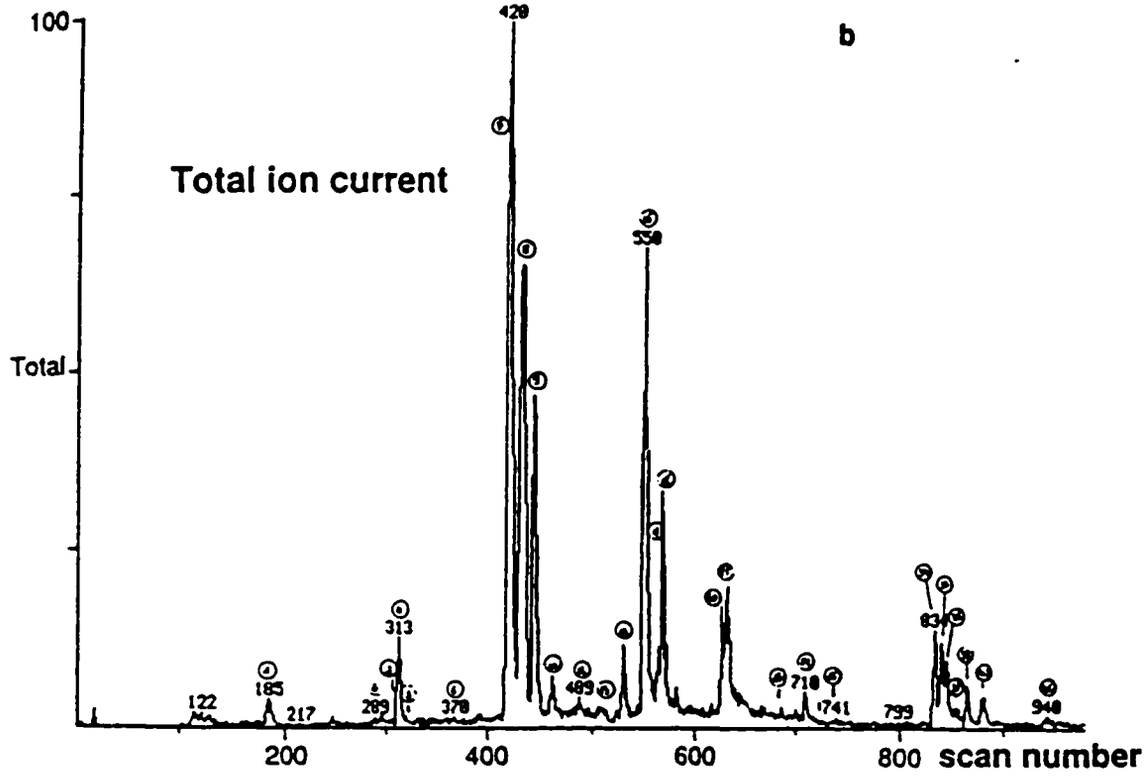


Figure 5.3.6. Total ion current of the elution of the proteolytic digest of rhIFN-γ (Reprinted with permission from ref. 143. Copyright (1993) Elsevier Science.)

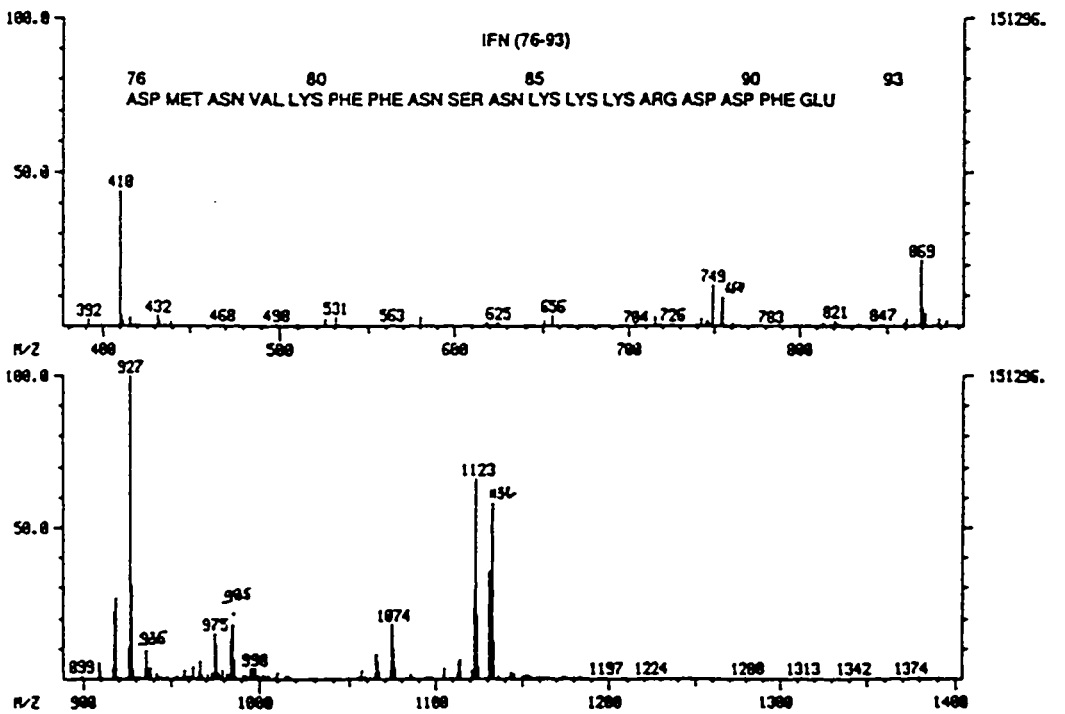


Figure 5.3.7. Mass spectrum of peak 21. The peak 21 in Fig. 5.3.6 is attributed to fragment IFN (76-93). (Reprinted with permission from ref. 143. Copyright (1993) Elsevier Science.)

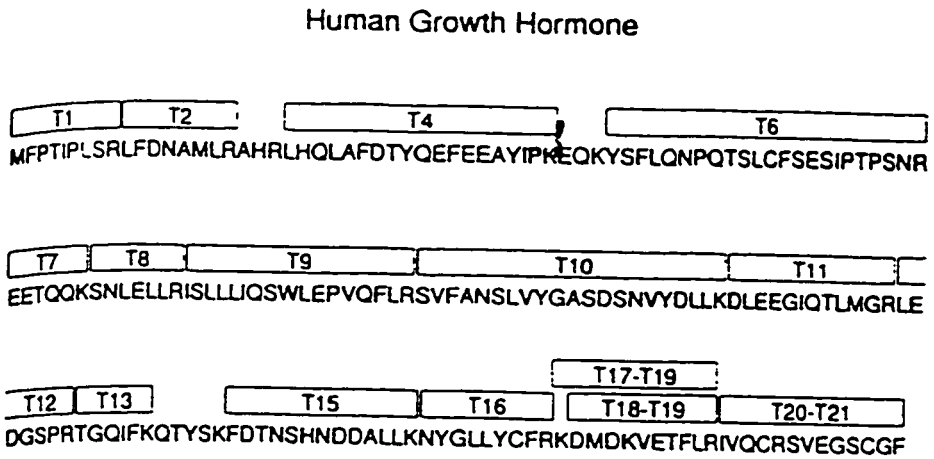


Figure 5.4.1. The amino acid sequence of met-rhGH and the observed tryptic peptides. (Reprinted with permission from ref. 146. Copyright (1990) Academic Press.)

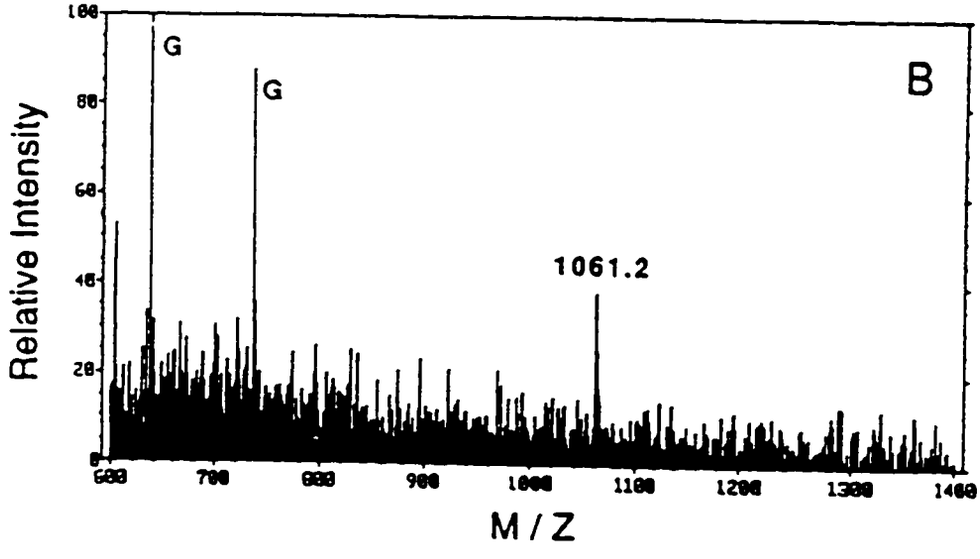
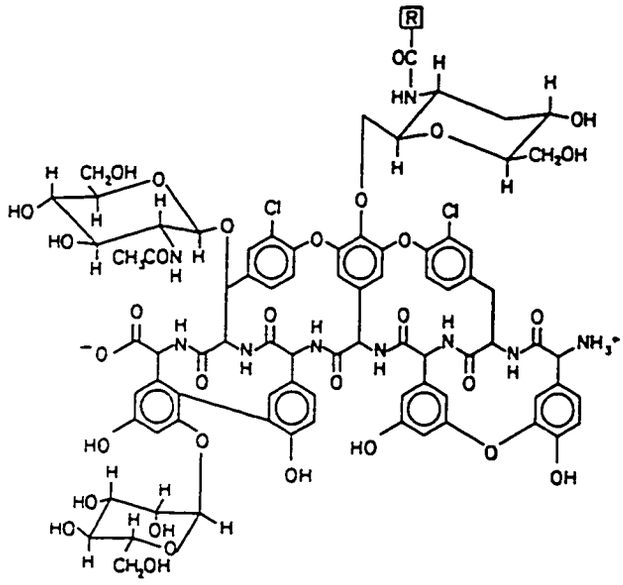


Figure 5.4.2. Mass spectrum for tryptic peptide fragment T_1 from a 500 fmol injection of trypsin digested met-rhGH. The peaks labeled "G" are protonated glycerol oligomers. (Reprinted with permission from ref. 146. Copyright (1990) Academic Press.)



Group	R	MH^+
A2-1	$CH_3(CH_2)_4CH{=}CH(CH_2)_2-$	1876.6
A2-2	$(CH_3)_2C(CH_2)_6$	1878.6
A2-3	$CH_3(CH_2)_8-$	1878.6
A2-4	$(CH_3)(C_2H_5)C(CH_2)_6$	1892.6
A2-5	$(CH_3)_2C(CH_2)_7$	1892.6

Figure 5.4.3. Structures of the teicoplanin A2 group. (Reprinted with permission from ref. 147. Copyright (1990) Elsevier Science.)

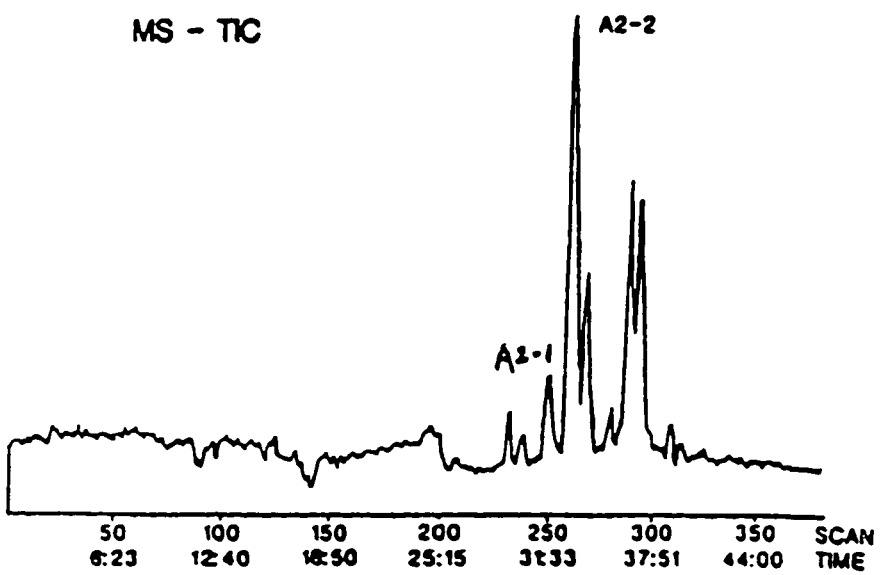


Figure 5.4.4. Mass total ion current (TIC) for teicoplanin. (Reprinted with permission from ref. 147. Copyright (1990) Elsevier Science.)

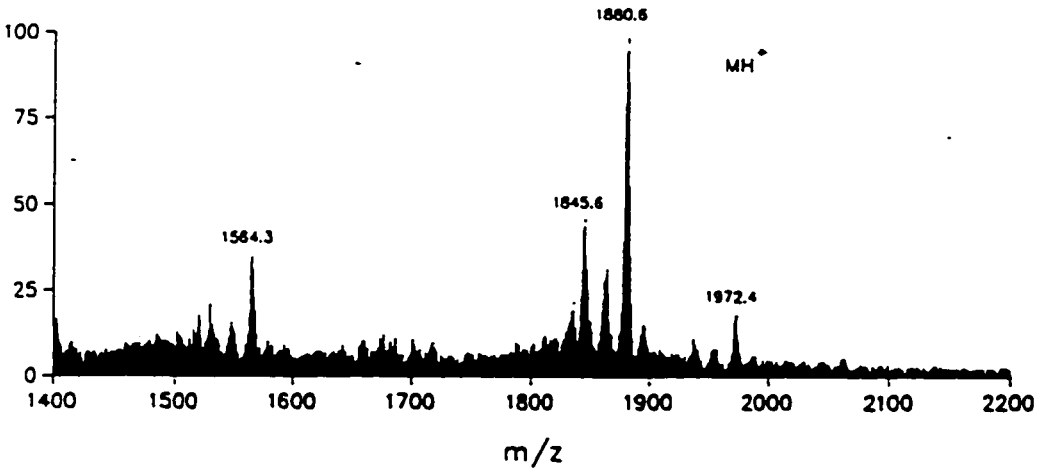


Figure 5.4.5. Mass spectrum of teicoplanin A2-1 (420 pmol). (Reprinted with permission from ref. 147. Copyright (1990) Elsevier Science.)

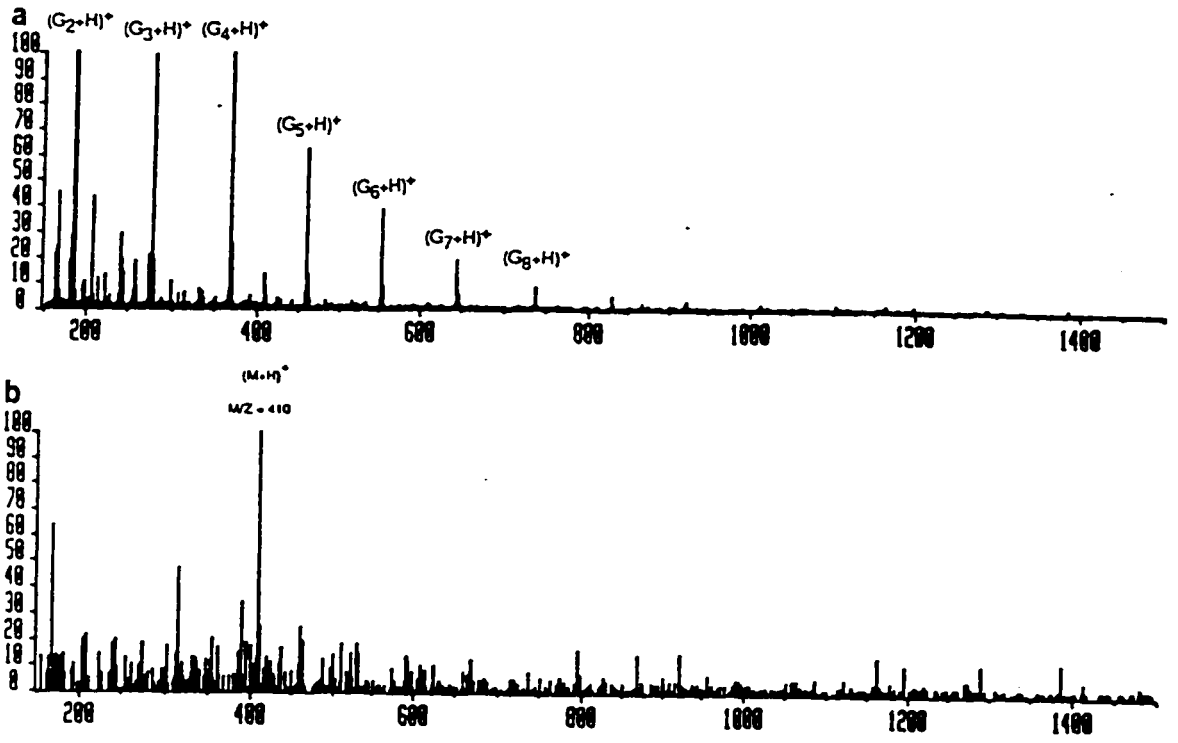


Figure 5.4.6. Mass spectrum of 54 fmol of Met-Leu-Phe. (a) raw data (G_nH^+ ions are matrix oligomers); (b) background subtracted data. (Reprinted with permission from ref. 148. Copyright (1989) American Chemical Society.)

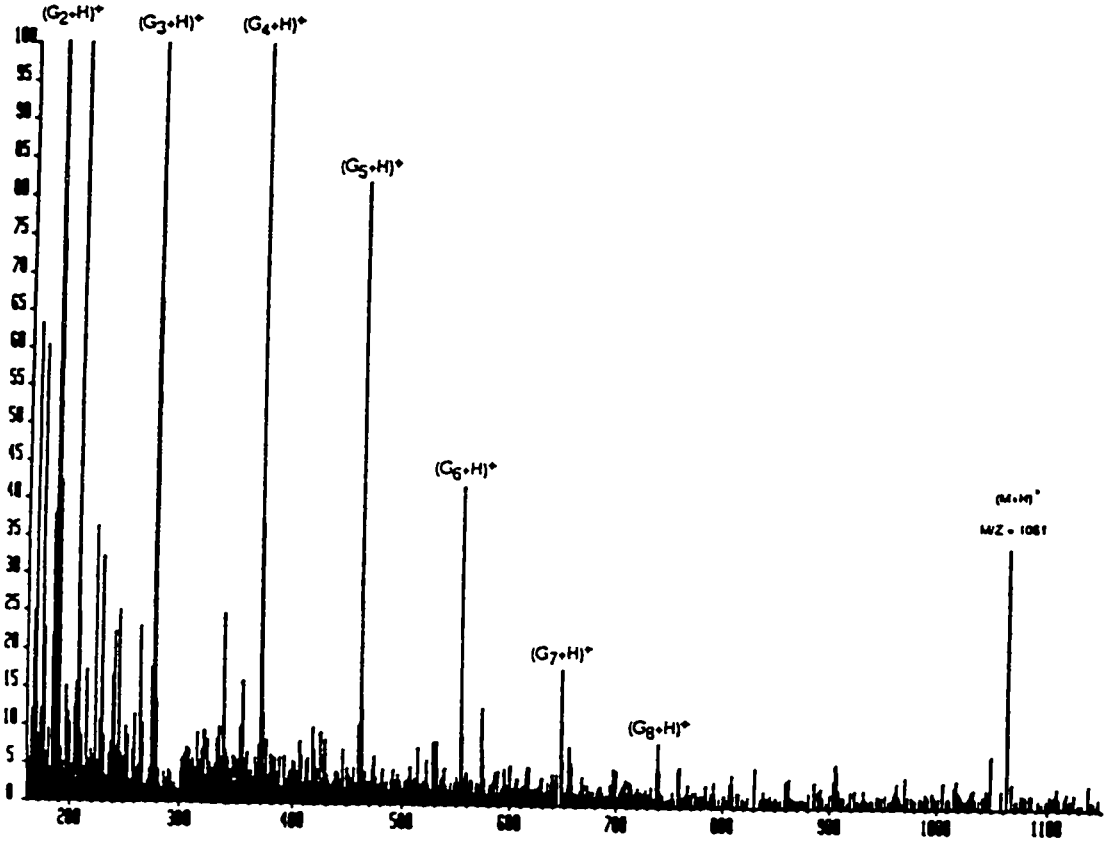


Figure 5.4.7. Mass spectrum of 850 fmol of bradykinin (G_nH^+ ions are matrix oligomers). (Reprinted with permission from ref. 148. Copyright (1989) American Chemical Society.)

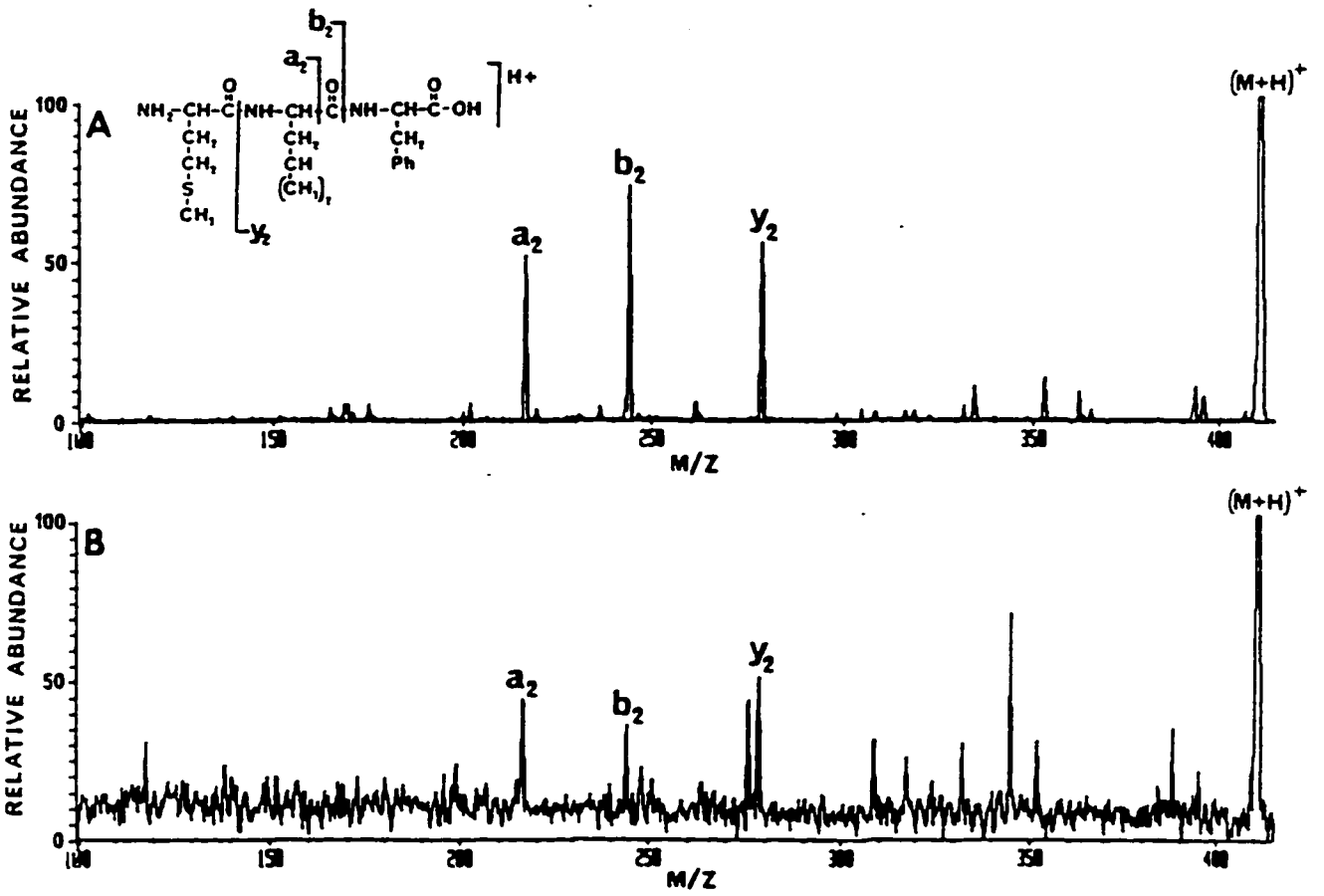


Figure 5.4.8. MS/MS spectra of (A) 220 pg and (B) 22 pg of the $(M + H)^+$ ion of Met-Leu-Phe. (Reprinted with permission from ref. 149. Copyright (1989) American Chemical Society.)

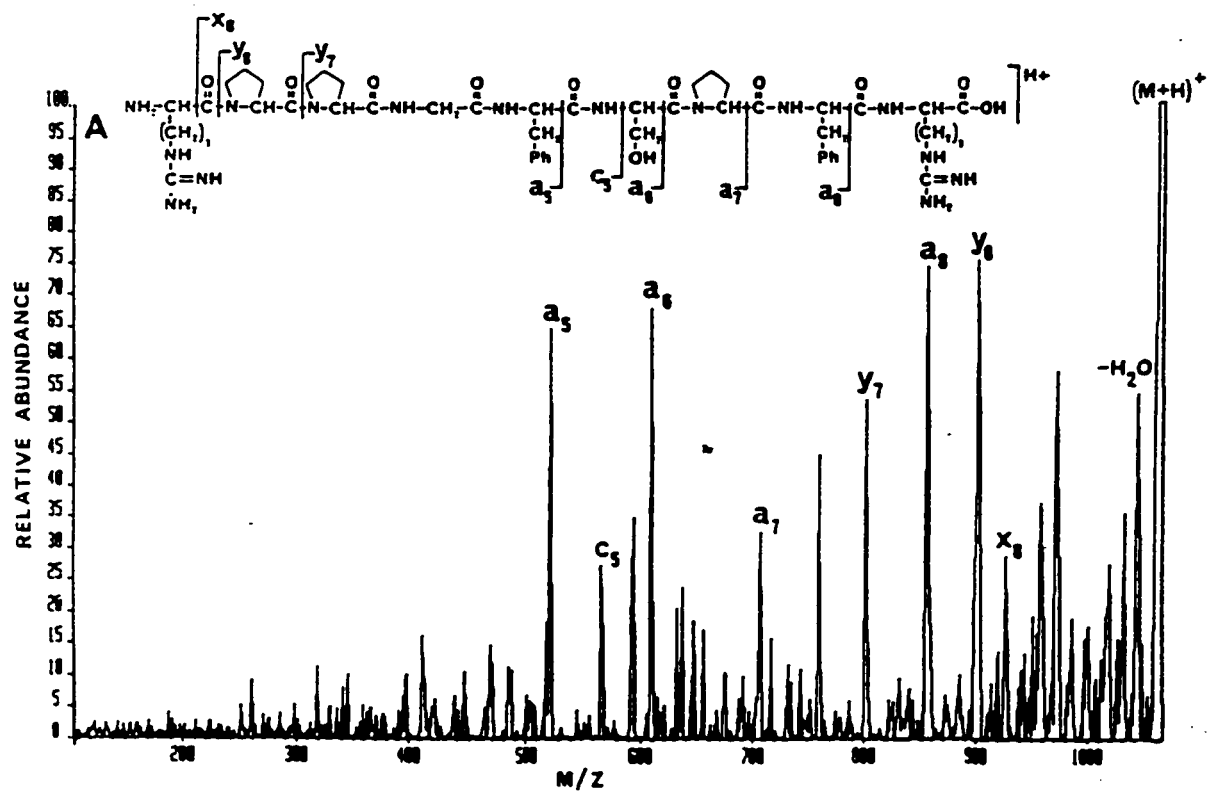


Figure 5.4.9. MS/MS spectrum of the $(M + H)^+$ ion of bradykinin. (Reprinted with permission from ref. 149. Copyright (1989) American Chemical Society.)

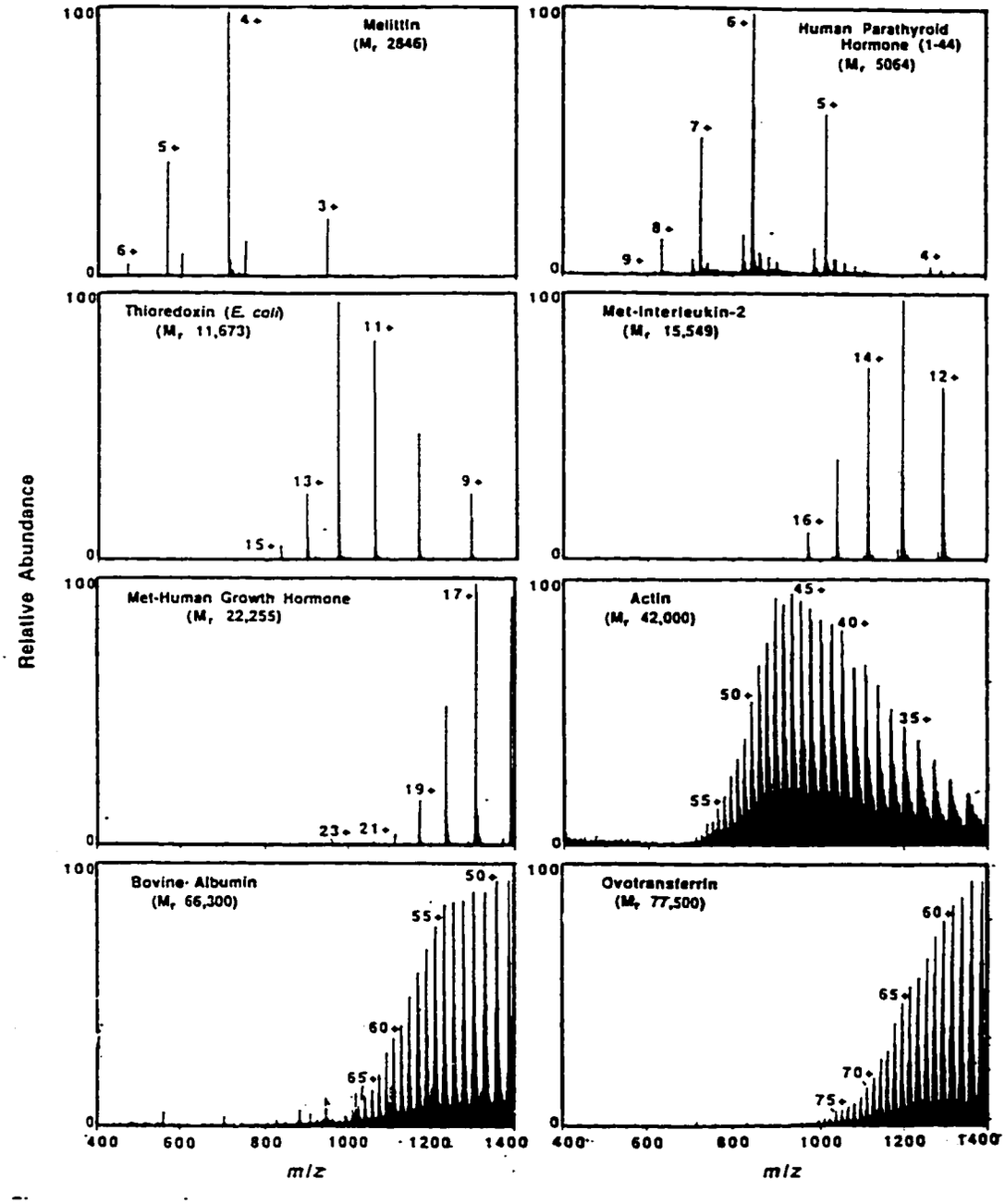


Figure 5.5.1. ESI mass spectra of eight peptides and proteins with M_r ranging from 2846 to 77,500. (Reprinted with permission from ref. 151. Copyright (1990) American Chemical Society.)

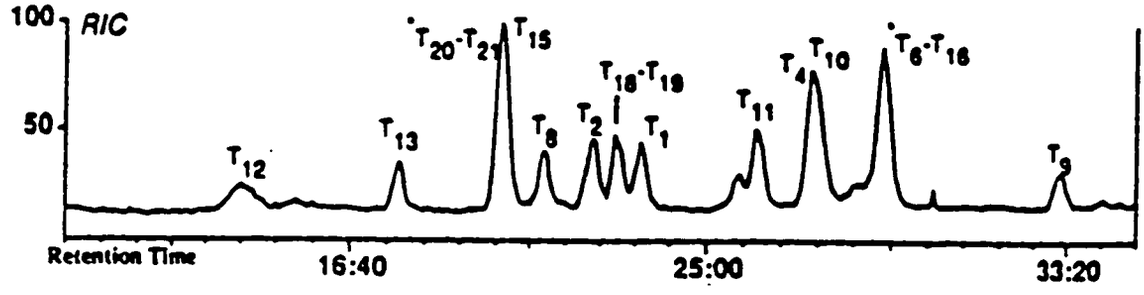


Figure 5.5.2. LC-ESI-MS of tryptic digest of rhGH. Retention time in min:s. (Reprinted with permission from ref. 158. Copyright (1993) Elsevier Science.)

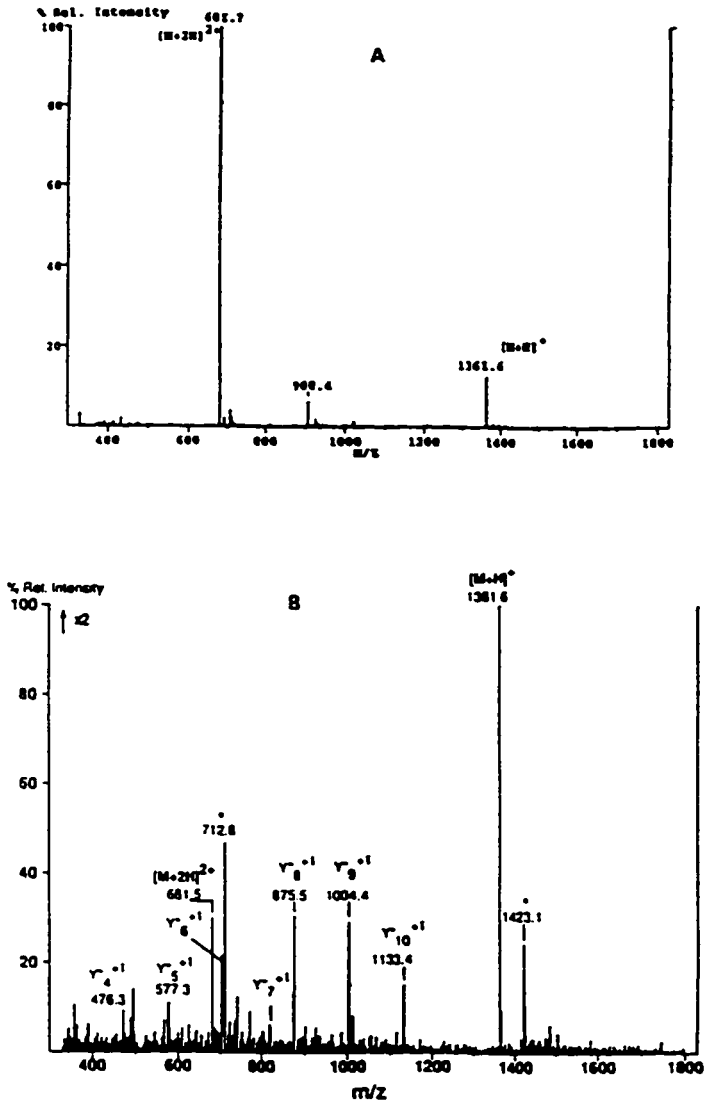


Figure 5.5.3. (A) Mass spectrum of tryptic fragment T_{11} of rhGH. Peptide sequence: DLEEGIQTLMGR. (B) Source-CAD spectrum of fragment T_{11} of rhGH obtained from an on-line LC-ESI-MS experiment. (Reprinted with permission from ref. 158. Copyright (1993) Elsevier Science.)

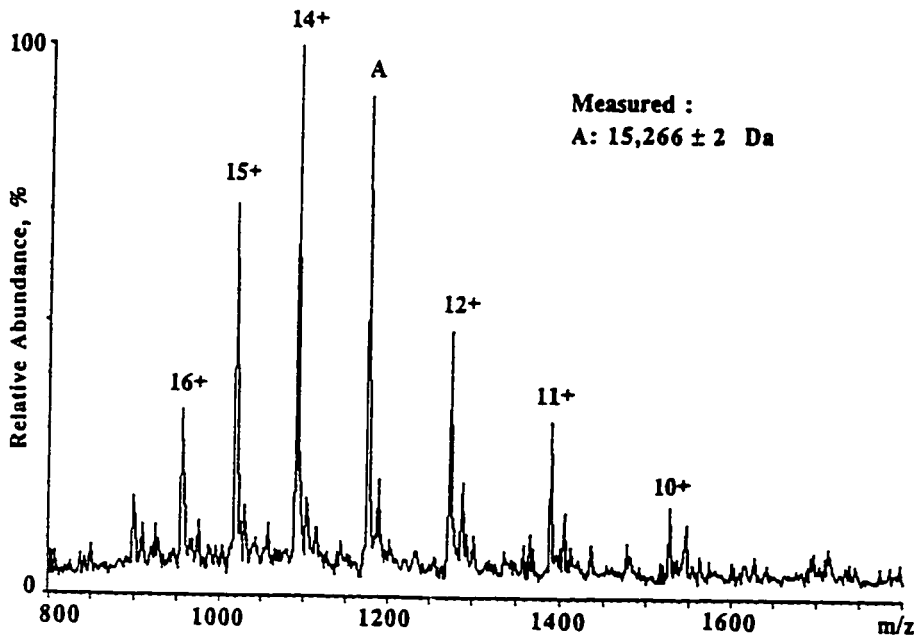


Figure 5.5.4. Electrospray mass spectrum of expressed rat liver microsomal cytochrome b_5 (expected mass: 15,266 Da). The protein was dissolved in 88% formic acid and injected into the carrier solvent which was CMV 2:5:2. (Reprinted with permission from ref. 159. Copyright (1993) Academic Press.)

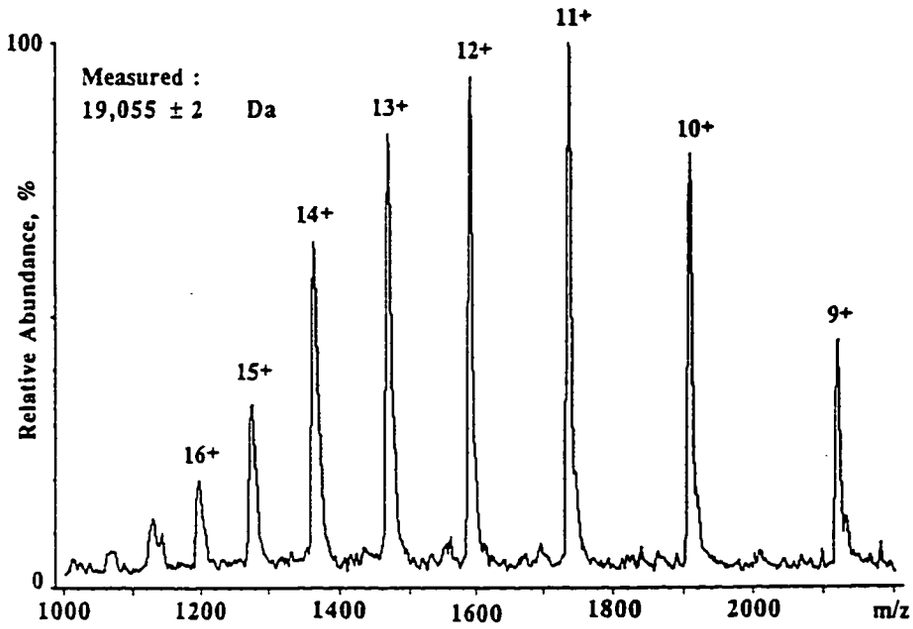


Figure 5.5.5. Electrospray mass spectrum of the C-terminal portion (C-1) of bacterioopsin expected mass: 19,056.3 Da). The protein was dissolved in the carrier solvent, CMW 2:5:2. (Reprinted with permission from ref. 159. Copyright (1993) Academic Press.)

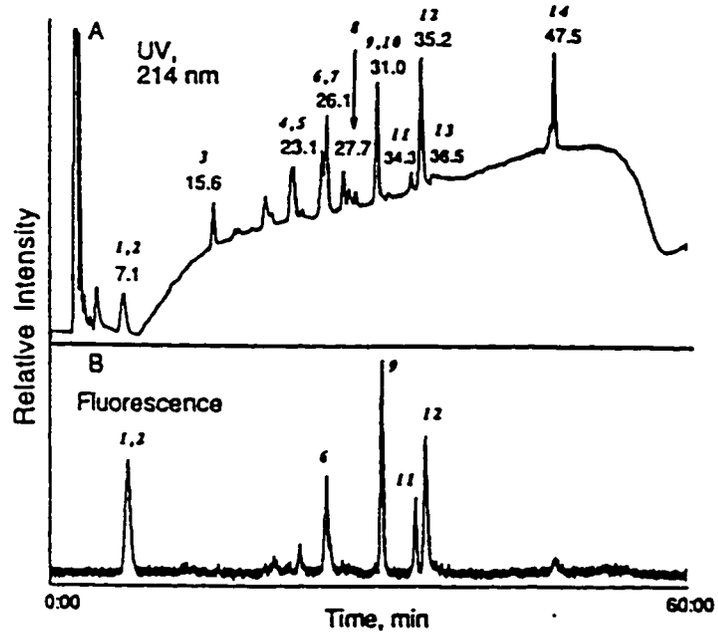


Figure 5.5.6. The (A) UV and (B) fluorescent chromatographic profiles of analysis of tryptic digest of expressed SCD. (Reprinted with permission from ref. 162. Copyright (1993) Elsevier Science.)

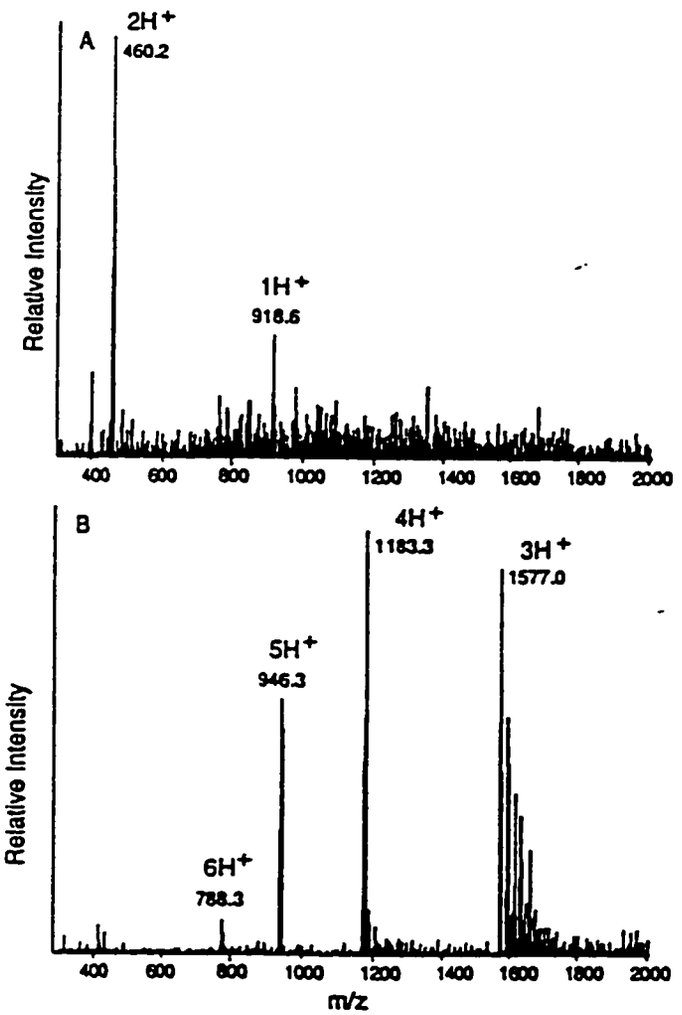


Figure 5.5.7. Electrospray mass spectra of a tryptic digest of expressed SCD for (A) peak 3 and has a M_r 917.5 and (B) peak 14 has a M_r 4726.4. (Reprinted with permission from ref. 162. Copyright (1993) Elsevier Science.)